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PRINCIPAL INVESTIGATOR: James D. Marks, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0962

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Marks, James D, M.D., Ph.D.

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San Francisco, California 94143-0962

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To produce neutralizing antibodies to Botulinum neurotoxin type A (BoNT/A), single chain Fv (scFv) phage antibody libraries were constructed from the immunoglobulin V_H and V_L genes of: 1) mice immunized with recombinant binding domain (H_C) of BoNT/A and boosted with BoNT/A; 2) mice immunized with BoNT/A H_C; 3) a human immunized with pentavalent BoNT toxoid, and 4) Non-immune humans. A total of 130 unique scFv were selected from the different phage antibody libraries which bound the different Botulinum serotypes. This included 51 scFv from immunized mice, 51 scFv from immunized humans and 28 scFv from the non-immune antibody library. Subsequent characterization focused primarily on those scFv which bound BoNT/A H_C since immunization with this domain has been shown to be protective in mice. A total of 44 unique scFv were identified that bound 15 different epitopes on BoNT/A H_C. scFv binding to each of these epitopes were evaluated in an *in vitro* toxin neutralization assay. scFv binding three of the epitopes were found to have toxin neutralizing capacity. Two of these neutralize toxin *in vivo* with enhanced potency when co-administered. The results indicate an approach to therapeutic toxin neutralization.

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1. Introduction

Botulinum toxins (BoNT) are extremely potent neurotoxins produced by anaerobic bacteria of the *Clostridia* genus. BoNT acts by inhibiting the release of acetylcholine at the neuromuscular junction causing a flaccid paralysis resulting in the disease Botulism. BoNT are among the most potent toxins known, with an LD_{50} in the range of 1 to 5 ng kg^{-1}. Strains of *C. botulinum* produce seven different BoNT, with toxin types A, B, E, and F being the main toxins that affect humans.

The toxin consists of disulfide linked heavy and light chain, with three major domains. The receptor binding site is at the carboxy terminus of the heavy chain (H_C or C-fragment). The binding domain mediates attachment to specific receptors on the presynaptic side of the synapse (1). Experimental evidence suggests both a ganglioside and protein receptor for the toxin (2). Internalization of the toxin occurs through receptor mediated endocytosis. The N-terminus of the heavy chain (H_N) is a channel forming domain, which permits the light chain to cross the membrane of the endocytic vesicle. The light chain is a zinc protease, which cleaves one of several proteins on the synaptosomal complex.

Botulism results from ingestion of the toxin, usually in food that has not been adequately sterilized. BoNTs have also been intentionally produced for use as biological warfare agents and weapons of terror due to their potency (microgram quantities are lethal) and ease of production using unsophisticated techniques For example, Iraq has declared the production of at least 19,000 liters of concentrated BoNT, of which 10,000 liters were loaded into munitions (3). Consequently, countermeasures designed to protect against this biological warfare agent have been developed, but each one has limitations. A polyvalent vaccine has been developed, but protective immunity takes months to develop and may be directed against only one or two of the seven distinct serotypes. Furthermore, vaccination requires identification of the population at risk. This is straightforward for battlefield troops, but impossible for civilians who could be exposed if the agent was used as a weapon of terror. Widespread immunization is also becoming less attractive since BoNTs are increasingly being used as a therapy for human diseases (4). Vaccination would deprive individuals of subsequent toxin therapy. Alternatively, neutralizing antibodies could be administered prophylactically or therapeutically to the population at risk. Polyvalent equine or human immune globulin protect experimental animals (5) and appear to protect humans (6) against BoNT intoxication. Immune globulin is most effective when administered prior to exposure, but can prevent disease up to 24 hours post exposure depending on the dose and route of exposure (5). With supportive care, immunoglobulin therapy reduces the duration of illness and cost of hospitalization. For example, a recently completed prospective randomized comparison of Human Botulinum Immunoglobulin (BIG) for infant Botulism indicated that treatment with BIG significantly decreased the duration of mechanical ventilation, length of hospital stay and cost of hospitalization (Dr. Steve Arnon (7) and unpublished results). These studies demonstrate the efficacy of passive immunotherapy, however equine immune globulin has a high incidence of side effects, including serum sickness and anaphylaxis (8). Human immune globulin should prove nontoxic, but requires a source of immunized human plasma donors.

Neutralizing monoclonal antibodies would provide an unlimited amount of antibody of defined specificity and reproducible titer. To date, however, no efficacious neutralizing monoclonal antibodies have been produced despite "years of effort by several fine laboratories" (5). Potential explanations for this failure include: 1) neutralizing epitope(s) are less immunogenic than other epitopes; 2) too few unique monoclonal antibodies have been studied; 3) use of a toxoid immunogen (formaldehyde inactivated crude toxin) which poorly mimics the conformation of the neutralizing epitope(s); 4) inadequate binding affinity of monoclonals produced to date for neutralization of a toxin with a high affinity ($K_d \sim 1$ nM) for its receptor; or 5) the need to block multiple epitopes to achieve efficient neutralization (8). Furthermore, murine monoclonal antibodies are not ideal therapeutics since they are immunogenic when administered to humans, resulting in decreased efficacy over time and the risk of allergic

reactions. Thus the ideal therapeutic would be neutralizing human monoclonal antibodies. In general, however, human monoclonal antibodies have proven extremely difficult to make using conventional hybridoma technology (9) and are frequently IgM and of low affinity.

For this contract, we proposed to use a novel technology, phage display, to produce neutralizing antibodies to Botulinum neurotoxin type A. The approach is reviewed below followed by results obtained.

1.1. Limitations of murine monoclonal antibodies

Production of monoclonal antibodies from hybridomas requires administration of an immunogenic antigen followed by harvesting of spleens and fusion with a suitable fusion partner. This process is relatively inefficient, leading to the production of a relatively few number of hybridomas. This makes it unlikely or impossible to produce monoclonal antibodies of certain rare specificities, for example neutralizing antibodies to BoNT/A. The affinities (K_d) of resulting monoclonal antibodies are also not likely to be better than 1.0×10^{-9} M (10). This K_d may not be adequate for therapeutic applications, such as neutralization of potent toxins such as BoNT/A.

A second disadvantage of murine antibodies is that they are likely to be immunogenic when administered therapeutically. Murine or chimaeric IgG are clearly immunogenic when administered to humans and some of the immune response is directed against the variable regions (11). Smaller size antibody fragments should be less immunogenic, but this still may be a problem when repeated doses are required for therapy. Thus therapeutic antibodies would ideally be of human origin. Unfortunately, production of human antibodies using hybridoma technology has proven extremely difficult (9).

The above limitations can be overcome by taking advantage of recent advances in biotechnology to produce human antibody fragments directly in bacteria, with or without prior immunization (reviewed in (12-14). Bacterial libraries containing millions to billions of human antibody fragments are created, from which binding antibody fragments can be selected by antigen. This approach makes it possible to examine every member of the library for binding, making it possible to 'screen' a vastly greater number of monoclonals than can be produced using conventional hybridoma technology. The affinities of the antibody fragments can also be increased *in vitro*, to values not achievable using conventional hybridoma technology.

1.2. Antibody nomenclature

Antibodies are bifunctional glycoproteins composed of domains of variable (V) and constant (C) sequence. The variable domains contain the regions that bind to antigen and consist of a light chain variable domain (V_L, either V_κ or V_λ) and a heavy chain variable domain (V_H). The V-domains can be classified into families based on DNA sequence homology. The smallest antigen binding unit is termed the Fv molecule (non-covalently linked V_H and V_L domains). Since the affinity of the V_H domain for the V_L domain is relatively low (micromolar), the two chains dissociate at typical concentrations. To overcome this limitation, the V_H and V_L domains can be linked together with a flexible peptide linker to create a single polypeptide chain, the single chain Fv (scFv) (15, 16). F_{ab} antibody fragments are composed of V_H-C_H1 and V_L-C_L domains covalently linked to each other by a disulfide bond. Each V_H and V_L domain consists of relatively conserved framework regions (FR1, FR2, FR3, and FR4) separated by three areas of hypervariable sequence called complementarity determining regions (CDR1, CDR2, and CDR3). The CDR's contain the majority of the antigen binding residues.

1.3. A new approach to making antibodies

The ability to express antibody fragments on the surface of viruses which infect bacteria (bacteriophage or phage) makes it possible to isolate a single binding antibody fragment from a library of greater than 10^{10} nonbinding clones. To express antibody fragments on the surface of phage (phage display), an antibody fragment gene is inserted into the gene encoding a phage surface protein (pIII) and the antibody fragment-pIII fusion protein is displayed on the phage

surface (17, 18). Since the antibody fragments on the surface of the phage are functional, phage bearing antigen binding antibody fragments can be separated from non-binding phage by antigen affinity chromatography (11). Depending on the affinity of the antibody fragment, enrichment factors of 20 fold - 1,000,000 fold are obtained for a single round of affinity selection. By infecting bacteria with the eluted phage, however, more phage can be grown and subjected to another round of selection. In this way, an enrichment of 1000 fold in one round can become 1,000,000 fold in two rounds of selection (17). Thus even when enrichments are low (19), multiple rounds of affinity selection can lead to the isolation of rare phage. Since selection of the phage antibody library on antigen results in enrichment, the majority of clones bind antigen after 4 rounds of selection. Thus only a relatively small number of clones (several hundred) need to be analyzed for binding to antigen. Analysis for binding is simplified by including an amber codon between the antibody fragment gene and gene III. The amber codon makes it possible to easily switch between displayed and soluble (native) antibody fragment simply by changing the host bacterial strain (18).

Phage display can be used to bypass conventional hybridoma technology after animal or human immunization. Repertoires of V_H and V_L genes are amplified from splenocytes or peripheral blood lymphocytes using primers optimized for the amplification of murine (20) or human (21, 22) immunoglobulin variable region genes. The V-genes are then cloned for expression as scFv or Fab antibody fragments on the surface of bacteriophage. Rare phage expressing binding antibody fragments are isolated by affinity selection as described above. Using this approach, murine and human antibodies have been made against tetanus toxin, hepatitis B surface antigen, and HIV-1 (22-24). Many different antibody fragments were isolated to each antigen, and the affinities compare favorably to the affinities of monoclonal antibodies produced using conventional hybridoma technology.

Human antibodies can be produced without prior immunization by displaying very large and diverse V-gene repertoires on phage (19). In the first example, natural V_H and V_L repertoires present in human peripheral blood lymphocytes were isolated from unimmunized donors by PCR. The V-gene repertoires were spliced together at random using PCR to create a scFv gene repertoire which was cloned into a phage vector to create a library of 30 million phage antibodies (19). From this single "naive" phage antibody library, binding antibody fragments have been isolated against more than 17 different antigens, including haptens, polysaccharides and proteins (19, 25, 26). The antibody fragments were highly specific to the antigen used for selection, and were functional in agglutination and immunofluorescence assays. With a library of this size and diversity, at least one to several binders can be isolated against a protein antigen 70% of the time (J.D. Marks, unpublished data). The antibody fragments are highly specific for the antigen used for selection and have affinities in the 1 uM to 100 nM range (19, 26). Larger phage antibody libraries result in the isolation of more antibodies of higher binding affinity to a greater proportion of antigens (27, 28).

Phage display is also an effective technique for increasing antibody affinity. Mutant scFv gene repertories, based on the sequence of a binding scFv, are created and expressed on the surface of phage. Higher affinity scFvs are selected by affinity chromatography on antigen as described above. One approach for creating mutant scFv gene repertoires has been to replace the original V_H or V_L chain with a repertoire of V-genes to create new partners (chain shuffling) (23). Using chain shuffling and phage display, the affinity of a human scFv antibody fragment which bound the hapten phenyloxazolone (phOx) was increased from 300 nM to 1 nM (300 fold) (29). Affinities of protein binding antibody fragments have been increased from 5 to 6 fold (into the nanomolar range) (30, 31), and more recently into the picomolar range (32).

1.4. Purpose of the present work and methods of approach

The purpose of this contract was to produce human monoclonal antibodies which neutralize BoNT/A for use as passive immunotherapy of Botulism or BoNT/A exposure or intoxication.

The proposed technical objectives were:

1. Construct an immune human scFv phage antibody library from the mRNA of volunteers immunized with polyvalent BoNT vaccine.
2. Isolate scFv antibody fragments which bind BoNT by selecting the immune scFv phage antibody library on immobilized BoNT and BoNT fragments.
3. Isolate scFv antibody fragments which bind BoNT by selecting a non-immune scFv phage antibody library on immobilized BoNT and BoNT fragments.
4. Characterize binding scFvs with respect to DNA sequence, specificity, cross reactivity, affinity, and kinetics of binding.
5. Increase the affinity of scFvs with the desired binding characteristics by creating mutant scFv phage antibody libraries and selecting on immobilized BoNT and BoNT fragments.
6. Characterize mutant binding scFvs with respect to DNA sequence, specificity, cross reactivity, affinity, and kinetics of binding.
7. Provide purified scFv protein to USAMRIID to perform *in vivo* protection studies.
8. If necessary, construct complete IgG1 human antibodies.

2. Body of report

2.1. Overview and Summary of Results

To maximize the chances of isolating neutralizing antibodies to BoNT/A, scFv phage antibody libraries were constructed from the V_H and V_L genes of: 1) mice immunized with recombinant binding domain (H_C) of BoNT/A and boosted with BoNT/A; 2) mice immunized with BoNT/A H_C; 3) a human immunized with pentavalent BoNT toxoid, and 4) Non-immune humans. BoNT/A H_C was used for murine immunization because it had been shown to elicit potent protection in mice (33). For human immunization, the only choice was pentavalent BoNT toxoid. Murine and human phage antibody libraries were selected on BoNT/A and BoNT/A H_C. Human libraries were also selected on BoNT/B, BoNT/C, and BoNT/E. Construction of these libraries met technical objectives 1 through 3. In addition, selection of the antibody libraries on the other toxin serotypes generated a large panel of monoclonal antibodies to serotypes not included in the original technical objectives. The murine library represented work begun under contracts DAMD17-93-C589 and DAMD17-94-M-5664 and was based on data obtained from USAMRIID that mice immunized with recombinant BoNT/A C-fragment were protected against subsequent exposure to BoNT/A. The murine libraries were deemed worthy of further characterization under the present contract and are included in this report.

Phage displaying binding scFv were identified by ELISA and unique scFv identified by DNA sequencing. BoNT specificity was determined by ELISA on BoNT/A, B, C, E. Domain specificity of anti-BoNT/A scFv was determined by ELISA on recombinant BoNT/A H_N and H_C. This work met technical objective 4.

A total of 130 unique scFv were selected from the different phage antibody libraries (Table 1). This included 51 scFv from immunized mice, 51 scFv from immunized humans and 28 scFv from the non-immune antibody library. The number of different scFv selected as well as their domain specificity are shown in Table 1.

The anti-BoNT/A scFv were selected for further characterization to identify monoclonal antibodies with toxin neutralizing capacity. Characterization focused primarily on those scFv which bound BoNT/A H_C since immunization with this domain has been shown to be protective in mice. Toxin neutralization was initially determined *in vitro* using a mouse hemidiaphragm preparation and measuring the time to 50% twitch tension reduction for BoNT/A alone and in the presence of 2.0×10^{-8} M scFv. These studies were performed at USAMRICD in the laboratory of Dr. Desphande (34). It was necessary to perform the initial screening for toxin neutralization *in vitro* because the small size (25 kDa) of the scFv leads to extremely rapid ($t_{1/2}$ = 2 hours) clearance from the circulation of mice and precluded the use of

the standard *in vivo* neutralization assay without modification of the scFv molecule to decrease clearance (see below). Such modification was not practical for evaluation of the large number of anti-BoNT/A scFv (44 unique scFv). The *in vitro* neutralization studies are also quite cumbersome, so it was decided to restrict the analysis to the highest affinity scFv which bound each unique (non-overlapping) epitope. Thus each scFv was epitope mapped using surface plasmon resonance in a BIAcore (35, 36) as described in (37) and the K_d for BoNT/A H_C measured as described in (37). This work met technical objective 4. The highest affinity scFv to each unique epitope was then studied for toxin neutralization in vitro. The results are summarized below in separate sections for immune murine, immune human and non-immune human libraries. This work met technical objective 7.

Table 1. Specificity of BoNT binding scFv selected from phage antibody libraries.

scFv Specificity	Number of unique scFv				
	mice immunized with BoNT/A H_C, boosted with BoNT/A	mice immunized with BoNT/A H_C	Humans immunized with toxoid	Non-immune humans	Total scFv
BoNT/A H_C	10	18	6	10	44
BoNT/A H_N	2	0	4	2	8
BoNT/A light chain	21	0	16	3	40
BoNT/B	ND	ND	16	5	21
BoNT/C	ND	ND	6	5	11
BoNT/E	ND	ND	3	3	6
Total	33	18	51	28	130

2.1A. Results from immune murine phage antibody libraries

51 unique scFv were obtained from immunized mice, 28 of these bound H_C. When mice were boosted with holotoxin (BoNT/A) prior to library construction, the immune response was directed away from H_C (only 10/33 bound H_C, 2 bound H_N, and the remainder bound the light chain). In mice not challenged with holotoxin prior to library construction, all scFv bound H_C. Only half of these bound BoNT/A, however, suggesting that a large portion of BoNT/A H_C is buried in the holotoxin. The 28 anti-BoNT/A H_C scFv recognized only 4 unique non-overlapping epitopes, with the majority of scFv (22/28) recognizing only 2 epitopes. Affinity, binding kinetics, and *in vitro* toxin neutralization were determined on one representative scFv binding to each epitope. For each epitope, the scFv chosen for further study had the best combination of expression level and slowest k_{off}, as determined during epitope mapping studies. K_d for the four scFv studied ranged between 7.3×10^{-8} M and 1.1×10^{-9} M (Table 2), values comparable to those reported for monoclonal IgG produced from hybridomas (38) C25 has the highest affinity ($K_d = 1.1 \times 10^{-9}$ M) reported for an anti-botulinum toxin antibody. *In vitro* toxin neutralization was determined using a mouse hemidiaphragm preparation. scFv binding to epitope 1 (S25) and epitope 2 (C25) significantly prolonged the time to neuroparalysis 1.5 fold (52%) and 2.7 fold (270%) respectively (Table 2 and Fig. 1). In contrast, scFv binding to epitopes 3 and 4 had no significant effect on the time to neuroparalysis. A mixture of S25 and C25 had a significant additive effect on the time to neuroparalysis, with the time to 50% twitch reduction increasing to 3.9 fold (390%). The majority of these results have been published (and see appendix 1) and are also detailed (below).

2.1B. Results from immune human phage antibody libraries

51 unique scFv were obtained from humans immunized with pentavalent Botulinum toxoid (26 anti-BoNT/A, 16 anti-BoNT/B, 6 anti-BoNT/C, and 3 anti-BoNT/E) (Table 1). All scFv were serotype specific, with no strain cross reactivity observed. Of the 26 anti-BoNT/A scFv, 6 bound H_C, 4 bound H_N, and 16 bound the light chain. The 7 anti-BoNT/A H_C scFv

recognized 3 non-overlapping H_C epitopes, with K_d ranging from 3.7×10^{-8} M to 8.0×10^{-9} M (Table 3)

Table 2. Affinities, binding kinetics, and *in vitro* toxin neutralization results of scFv selected from phage antibody libraries.

scFv clone	Epitope	K_d[a] (M)	k_{on} ($\times 10^4$ M^{-1} s^{-1})	k_{off} ($\times 10^{-3}$ s^{-1})	Paralysis Time[b]
S25	1	7.3×10^{-8}	1.1	0.82	85 ± 10[c]
C25	2	1.1×10^{-9}	30	0.33	151 ± 12[c]
C39	2	2.3×10^{-9}	14	0.32	139 ± 8.9[c]
1C6	3	2.0×10^{-8}	13	2.5	63 ± 3.3
1F3	4	1.2×10^{-8}	92	11	52 ± 1.4
C25 + S25 Combination					218 ± 22[c]
BoNT/A pure toxin (control)					56 ± 3.8

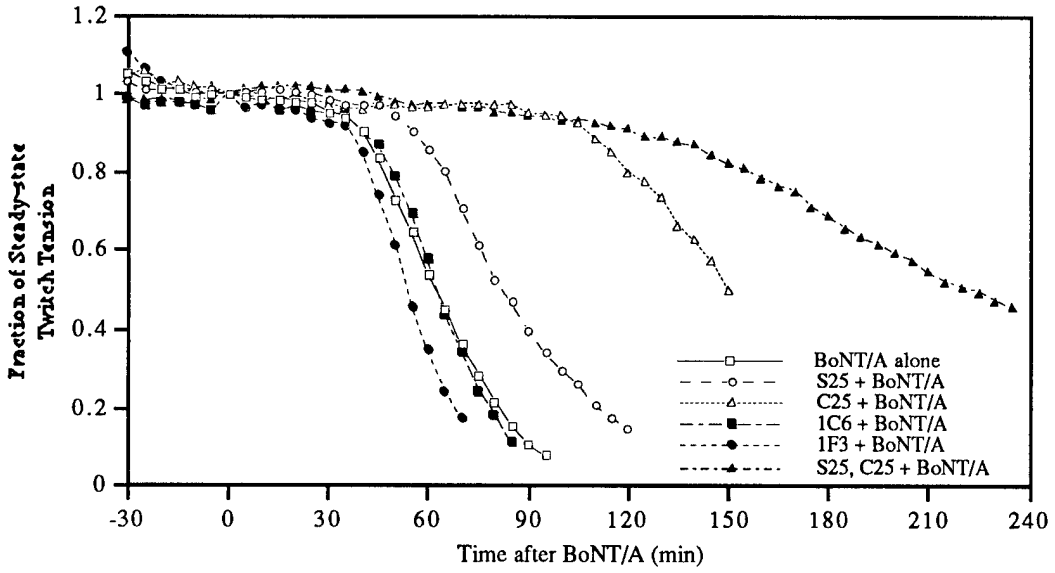
[a] k_{on} and k_{off} were measured by surface plasmon resonance and K_d calculated as k_{off}/k_{on}.

[b] Time (min.) to 50% twitch reduction in mouse hemidiaphragm assay using 20 nM scFv + 20 pM BoNT/A, compared to time for BoNT/A alone. Each value is the mean ± SEM of at least three observations.

[c] $p < 0.01$ with respect to BoNT/A.

[d] $p < 0.05$ compared to C25.

Figure 1. Evaluation of murine scFv neutralization of BoNT/A in a mouse hemidiaphragm model.



The epitopes recognized by scFv from the immune human phage antibody library were compared to epitopes recognized by scFv from murine antibody libraries using surface plasmon resonance in a BIAcore. The 3 BoNT/A H_C epitopes recognized by the human scFv did not overlap the epitopes recognized by scFv selected from the murine antibody library. scFv binding to epitope 4 (3D12) significantly prolonged the time to neuroparalysis 1.33 fold (33%) (Table 3 and figure 2). While non-overlapping, epitope 4 is located near to epitope 1 (recognized by the murine scFv S25). Similar to results observed with S25, a mixture of 3D12 and the murine scFv C25 had an additive effect on neutralization (Table 3 and Figure 2). 3F10 binding to epitope 5 had no effect on time to neuroparalysis. 2B11 was not studied for in vitro neutralization due to extremely low expression levels.

Table 3. Affinities, binding kinetics, and in vitro toxin neutralization results of scFv selected from phage antibody libraries.

scFv clone	Epitope	K_d[a] (M)	k_{on} ($\times 10^5\ M^{-1}\ s^{-1}$)	k_{off} ($\times 10^{-3}\ s^{-1}$)	Paralysis Time[b]
Immune Library					
3D12	4	3.69×10^{-8}	0.13	0.50	85 ± 5.0[c]
3F10	5	7.80×10^{-9}	0.80	0.62	55 ± 5.0
2B11	6	ND	ND	ND	ND
C25 + 3D12 Combination					179 ± 2.3[c]
BoNT/A pure toxin (control)					63.9 ± 2.3
Non-immune Library					
2A2	7	1.98×10^{-7}	2.35	46.7	56.3± 9.7
2B10	8	1.29×10^{-7}	5.57	71.6	62.3± 6.7
2E6	9	1.93×10^{-7}	1.19	23.0	60.9 ± 8.2
2H6	10	3.86×10^{-8}	2.20	8.50	63.0± 5.0
3G11	11	1.07×10^{-7}	0.83	8.88	58.4± 4.0
2A9	12	2.61×10^{-8}	0.25	0.66	71.0± 3.0
2B6	13	7.15×10^{-8}	1.09	7.80	61.9± 5.0
3F6	14	6.60×10^{-8}	4.69	30.9	60.4± 3.6
3C2	15	3.90×10^{-8}	2.10	82.0	61.9± 4.8

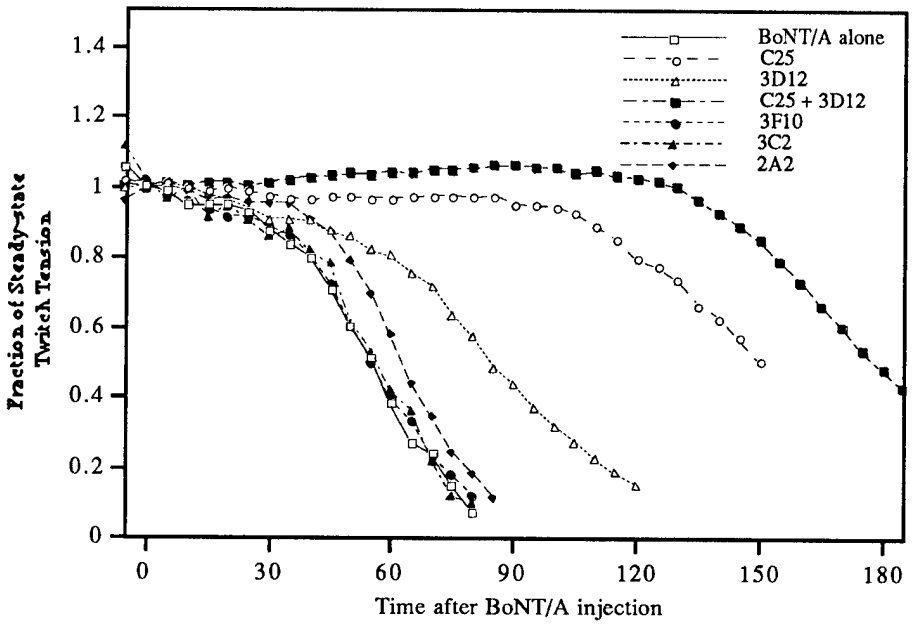
[a] k_{on} and k_{off} were measured by surface plasmon resonance and K_d calculated as k_{off}/k_{on}

[b] Time (min.) to 50% twitch reduction in mouse hemidiaphragm assay using 20 nM scFv + 20 pM BoNT/A, compared to time for BoNT/A alone. Each value is the mean ± SEM of at least three observations.

[c] $p<0.01$

ND: Not determined.

Figure 2. Evaluation of immune and non-immune human scFv neutralization of BoNT/A in a mouse hemidiaphragm model.



2.1C. Results from non-immune human phage antibody libraries

A 7.0 x 10^9 member human scFv phage antibody library was created in pHEN-1 by creating separate V_H and V_L libraries on separate replicons, combining them into an scFv gene repertoire by splicing by overlap extension, and cloning the scFv gene repertoire into the phage display vector pHEN1 ((39) and see appendix 2). 27 unique scFv were isolated from the non-immune human phage antibody library (14 α-BoNT/A, 5 α-BoNT/ B, 5α-BoNT/ C, and 3 α-BoNT/E (see Table 1). After selecting on BoNT/A H_C, half of the scFv analyzed (10 unique scFv) bound both BoNT/A H_C and the holotoxin. The 10 scFv bound to 9 different epitopes, with K_d ranging from 2.0 x 10^{-7} M to 3.8 x 10^{-8} M (Table 3). The epitopes recognized did not overlap epitopes recognized by scFv selected from immune murine and human phage antibody libraries.

In vitro toxin neutralization was determined using a mouse hemidiaphragm preparation for scFv binding to each non-overlapping epitope on H_C. None of the scFv from the non-immune library neutralized BoNT/A *in vitro* (Table 3 and figure 2, above).

2.1D. Determination of in vivo toxin neutralization capacity of monoclonal antibodies

To determine the in vivo neutralization capacity of scFv, it was necessary to modify the molecules to prolong the serum half life. To do this, we decided to fuse the scFv to the Fc portion of human IgG1. The resulting scFv-Fc fusion would have a molecular mass of approximately 100 kDa, well above the renal threshold for clearance. The expression host we chose was the methyltrophic yeast *Pichia pastoris*. We chose *Pichia* due to its reported high expression levels of recombinant protein and the relative speed with which the fermentations could be performed (three to five days compared to several weeks for mammalian expression systems). Construction of scFv-Fc fusions also greatly reduced the time necessary to create the genetic constructs for expression. The scFv gene could be simply subcloned intact into the appropriate expression vector. Construction of complete IgG would require subcloning both the V_H and V_L genes separately. Pichia vectors for co-expression of two chains (as required for IgG) also do not exist, so we would have had to use a mammalian system. Since we wanted to analyze a relatively large number of scFv, it was decided to construct scFv-Fc fusions and use Pichia as the expression host.

To date, scFv-Fc fusions have been constructed for the two murine scFv which showed neutralization capacity in the hemidiaphragm assay (S25 and C25) as well as for the human scFv 3D12 which also showed neutralization capacity in the same assay. Both C25 and S25 were expressed in shake flasks with yields after purification of 1.5 mg/L for C25 and 300 ug/L for S25. 3D12 has yet to be expressed. To determine the pharmacokinetics of scFv-Fc fusions in mice, the C25-Fc fusion protein was radiolabelled and administered to mice in 20 ug doses, both intravenously and intraperitoneally. Figure 3 shows that the C25-Fc fusion had dramatically prolonged serum perseverance whether administered intravenously or intraperitoneally, with a $t_{1/2}$ for the beta phase of 52 and 93 hours for i.v. or i.p. administration respectively. This compares to only 2.5 to 3.5 hours for an scFv (40). The increased retention of the scFv-Fc fusions can be attributed to the increased size of the scFv-Fc homodimer which places the mass well above the renal threshold for clearance.

To determine neutralization capacity *in vivo*, mice were injected intraperitoneally either with 20 or 100 LD50s of BoNT/A alone or mixed with 50 ug of either C25 scFv-Fc or S25 scFv-Fc or a combination of C25 and S25 scFv-Fc fusions. The results are shown in figure 4. At the lower does of toxin (20 LD50s), there was significant prolongation of the time to death. Without antibody, all mice were dead by 24 hours. In contrast 6/6 mice receiving toxin plus S25 fusion were alive and 4/6 mice receiving C25 were alive. Survival decreased with increasing time from antibody and toxin administration. At the higher dose of toxin (100 LD50s), only a minor prolongation is seen with either C25 or S25 scFv-Fc fusions compared to toxin alone (figure 4).

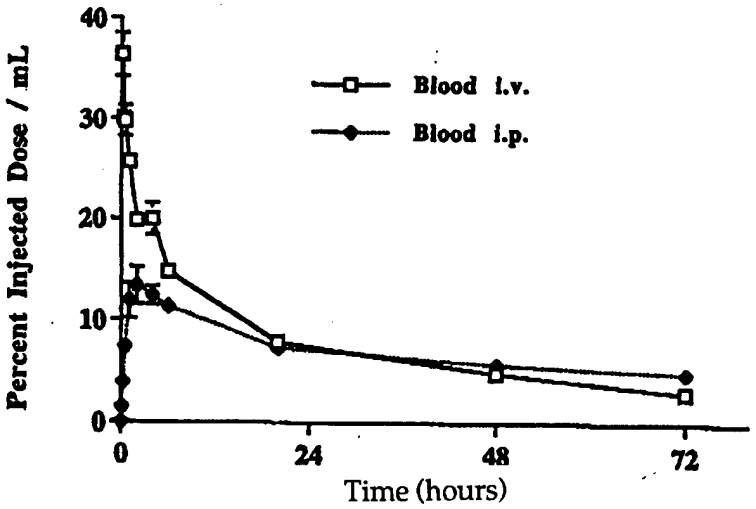


Figure 3. Biodistribution of scFv-Fc fusions injected into mice either intravenously or intraperitoneally.

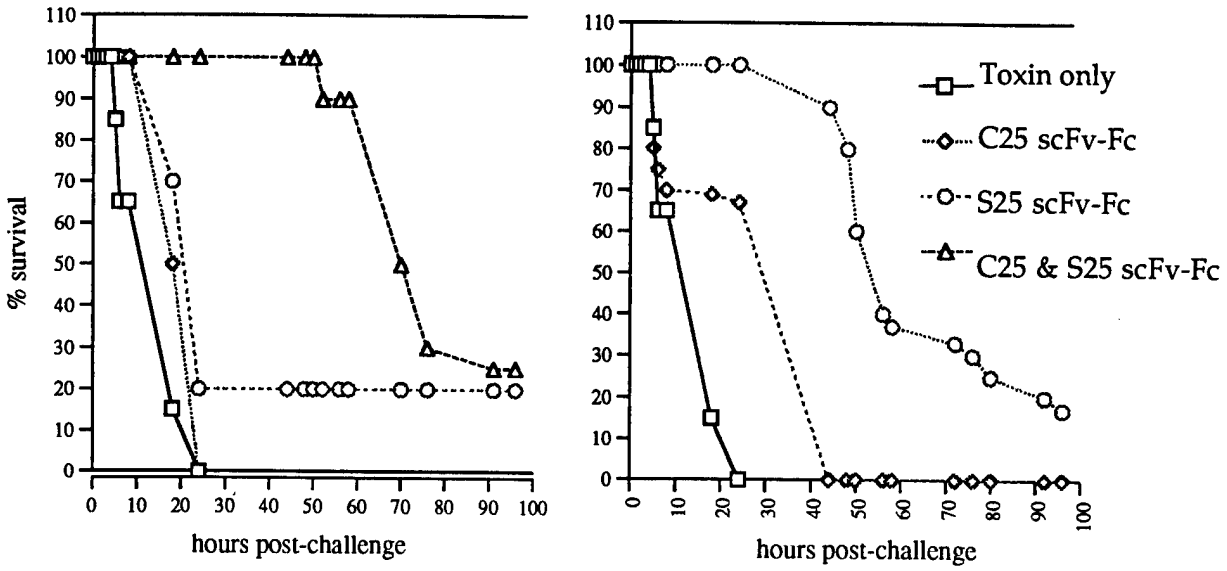


Figure 4. Results of in vivo toxin neutralization by C25 and S25 scFv-Fc fusions. Mice were injected intraperitoneally (i.p.) with 20 or 100 LD50s of toxin or toxin plus one or both of the scFv-Fc fusions. Left panel = 20LD50s of toxin, right panel = 100 LD50s of toxin.

When C25 and S25 are administered together, time to death is prolonged significantly at the higher toxin does, with one mouse surviving at 24 hours. The results indicate that both C25 and S25 have *in vivo* toxin neutralization capacity, albeit at relatively modest toxin doses. It is likely that this effect would be greater if the serum concentrations of the antibodies were higher. As can be seen from the biodistribution data, the serum concentrations of antibody at the time the mice begin dying (48 hours) is less than 10% of the administered dose. For a complete IgG, this value would be closer to 50%. We hypothesize that the more rapid clearance of the Fc fusion results from the glycosylation pattern in *Pichia pastoris*. *Pichia* hypermannosylates proteins, which can then be rapidly cleared *in vivo* by the high affinity mannose receptors. Analysis of the scFv-Fc fusions before and after deglycosylation indicates that the majority of the protein is glycosylated. Removal of the glycosylation site in the Fc should lead to a significantly higher serum level of antibody at the later time points. This could lead to a significant increase in the survival rate observed. Co-administration of S25 and C25 lead to partial protection against higher toxin doses. This recapitulates *in vitro* toxin neutralization

results (figure 1) showing a synergistic effect of the C25 and S25 combination in toxin neutralization. Again, it is likely that this effect would be more marked with higher serum concentrations of antibody. Increasing antibody affinity should also lead to more potent toxin neutralization. In section 4 (conclusions) the implications of these results are discussed.

2.2 Detailed results

In the sections below, we provide more details of the results summarized above. This section is followed by conclusions and our future plans.

2.2A. Results from immune human scFv phage antibody libraries

2.2A1. Construction of immune human scFv phage antibody libraries from the mRNA of volunteers immunized with polyvalent BoNT vaccine.

Eight previously immunized human volunteers were boosted with pentavalent Botulinum toxoid at USAMRIID and peripheral blood harvested two weeks later. Peripheral blood lymphocytes were separated over Ficoll and total RNA prepared. This RNA was used as the source material for preparation of human immune phage antibody libraries. The V_H and V_L genes were amplified from the RNA of all 8 donors using pools of family specific V_H, V_κ, and V_λ Back primers and J_H, J_κ, and J_λ Forward primers (19, 21)). The V_H and V_L genes of donor 6 were spliced together to create an scFv gene repertoire. The scFv gene repertoire was digested with NcoI and NotI and ligated into pCANTAB5E (Pharmacia) digested with NcoI and NotI. Aliquots of the ligation mixture were used to transform electrocompetent *E. coli* TG1 to create a phage antibody library of 7.7×10^5 transformants. PCR screening was used to determine the percent of clones with proper size insert. By PCR screening, 15/15 randomly selected clones had full length scFv insert by PCR screening (figure 1A), 66% of these have a V_κ light chain and 33% have a V_λ light chain (as determined by PCR screening, data not shown). BstN1 fingerprinting was used to determine the extent of library diversity (23). All 15 scFv screened had a unique BstN1 fingerprint (figure 5B). The number of clones capable of secreting scFv was determined by dot blotting using a monoclonal antibody directed against the E-tag at the C-terminus of scFv. 55% of randomly selected clones expressed scFv as determined by dot blotting with anti-E tag antibody (figure 6). 5 random clones were sequenced and all had human V_H and V_L genes (data not shown). This phage antibody library prepared from donor 8 was selected on BoNT/A, BoNT/B, BoNT/C, BoNT/E, and BoNT C-fragment.

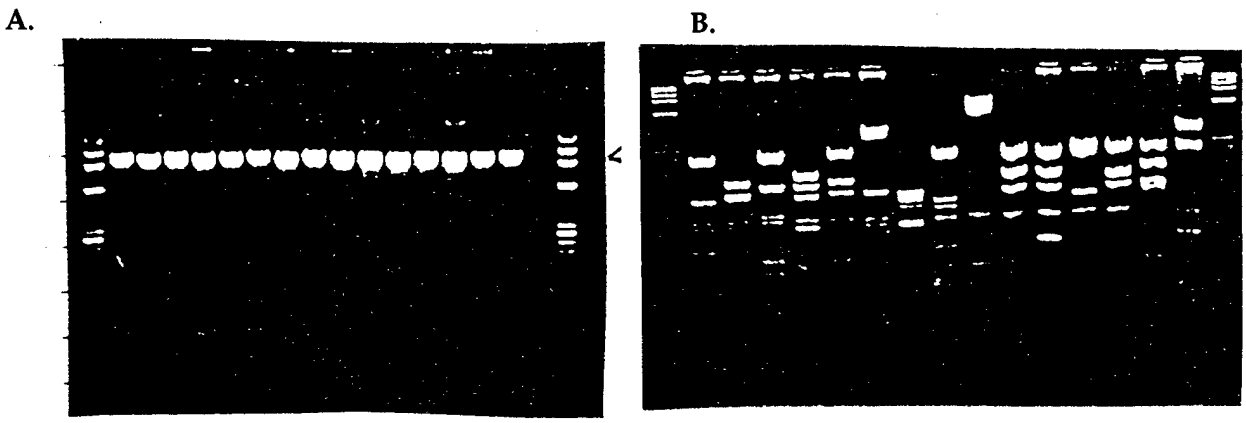


Figure 5. PCR screening and fingerprint analysis of an scFv phage antibody library prepared from human donor 6 immunized with pentavalent Botulinum toxoid. A. Agarose gel analysis of the results of PCR screening. All 15 clones screened had an scFv size insert (approximately 800 bp). B. Results of BstN1 fingerprinting. The PCR product shown in panel A was digested with BstN1 and the products analyzed on an agarose gel. All 15 clones analyzed had a unique fingerprint, demonstrating the diversity of the library at the DNA level.

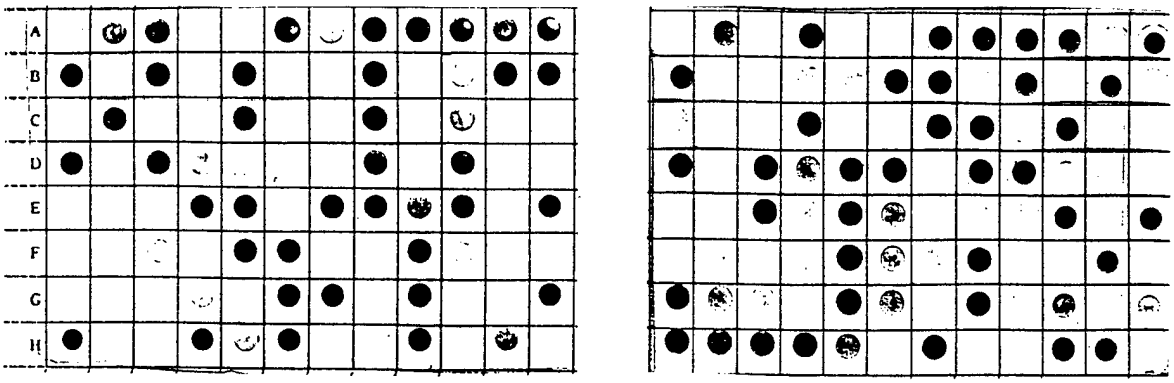


Figure 6. Evaluation of the scFv expression level of randomly selected clones from an scFv phage antibody library prepared from human donor 6 immunized with pentavalent Botulinum toxoid. scFv expression was induced from 96 randomly selected clones from an immune human scFv phage antibody library at either 30°C (left panel) or 25°C (right panel). Bacterial supernatant containing expressed scFv was applied to a nitrocellulose membrane, and the presence of scFv detected using an anti-E tag antibody and anti-mouse Fc-HRP. Best expression results were obtained at 25°C, with 48/96 (50%) expressing detectable quantities of scFv.

2.2A2. Selection of BoNT binding scFv from an immune human library

Phage expressing scFv which bound Botulinum neurotoxins were selected by panning the phage library on immobilized BoNT/A, BoNT/B, BoNT/C, BoNT/E, or BoNT C-fragment. Toxins were provided either by Dr. Ray Stevens (BoNT/A) or USAMRIID (BoNT/B, BoNT/C, BoNT/E) and recombinant C-fragment expressed from *E. coli* was obtained from Ophidian. Briefly, immunotubes (Nunc, Maxisorb) were coated with 2 ml (50 µg/ml) of BoNT/A, BoNT/B, BoNT/C, BoNT/E, or BoNT C-fragment in PBS overnight at 20°C and blocked with 2% milk powder in PBS for 2 h at 37°C. 1 ml of the phage solution (approximately 10^{13} phage) was added to the tubes and incubated at 20°C for 2 hours. Nonbinding phage were eliminated by sequential washing (15 times with PBS containing 0.05% Tween followed by 15 times with PBS). Binding phage were then eluted from the immunotubes by adding 1ml of 100 mM triethylamine, incubating for 10 min at 20°C, transferring the solution to a new tube, and neutralizing with 0.5 ml 1M Tris HCl, pH 7.4. Half of the eluted phage solution was used to infect 10 ml of *E.coli* TG1 grown to an A_{600} nm of 0.8-0.9. After incubation for 30 min at 37°C, bacteria were plated on TYE plates containing 100 µg/ml ampicillin and 1% glucose and grown overnight at 37°C. Phage were rescued and concentrated by precipitation with PEG and used for the next selection round. The selection process was repeated for a total of 3 rounds.

2.2A3. Screening for BoNT binding scFv from an immune human library

After each round of selection, 10 ml of *E.coli* HB2151 (A_{600} nm ~ 0.8) were infected with 100 µl of the phage eluate in order to prepare soluble scFv. In this strain, the amber codon between the scFv gene and gene III is read as a stop codon and native soluble scFv secreted into the periplasm and media (18). Single ampicillin resistant colonies were used to inoculate microtitre plate wells containing 150 µl of 2 x TY containing 100 µg/ml ampicillin and 0.1% glucose. The bacteria were grown to an A_{600} nm of 1.0, and scFv expression induced by the addition of IPTG to a final concentration of 1 mM (19). Bacteria were grown overnight at 30°C, the cells removed by centrifugation, and the supernatant containing scFv used directly in an ELISA to detect binding.

To screen for binding, 96-well microtiter plates (Falcon 3912) were coated overnight at 4°C with 50 µg/ml BoNT/A, BoNT/B, BoNT/C, BoNT/E, or BoNT C-fragment in PBS, blocked for 2 h at 37°C with 2% milk powder in PBS, and incubated for 1.5 hours at 20°C with 50 µl of

the *E.coli* supernatant containing scFv. Binding of soluble sFv to antigen was detected with a mouse monoclonal antibody which recognizes the C-terminal E peptide tag and peroxidase conjugated anti-mouse Fc antibody (Sigma) using ABTS as substrate. The reaction was stopped after 30 min with NaF (3.2 mg/ml) and the A405 nm measured. The number of unique clones was identified by PCR fingerprinting (as described above) followed by DNA sequencing of the V_H and V_L genes of at least two clones of each fingerprint pattern. The specificity of each unique scFv was determined by ELISA performed as described above with wells coated with 50 µg/ml of BoNT/A, BoNT/B, BoNT/C, BoNT/E, BoNT C-fragment, as well as irrelevant proteins.

2.2A4. Results of selection and screening from an immune human library

Results of selections performed on BoNT A, BoNT B, BoNT C, and BoNT E are shown in Table 4. For each BoNT serotype, the titre of eluted phage increased after each round of selection. After the third round of selection, 43% to 80% of scFv bound the antigen used for selection.

Table 4. Results of selection of immune scFv phage antibody library on BoNT/A, BoNT/B, BoNT/C, and BoNT/E.

Antigen used for selection	Round of selection	Eluted phage titre	ELISA positive scFv
BoNT type A	1	2.0×10^4	0/92
	2	5.0×10^4	27/92
	3	1.7×10^6	73/92
BoNT type B	1	8.1×10^5	1/92
	2	4.0×10^6	27/92
	3	5.4×10^8	63/92
BoNT type C	1	2.6×10^5	ND
	2	1.6×10^6	ND
	3	1.2×10^8	40/92
BoNT type E	1	2.6×10^5	ND
	2	1.6×10^6	ND
	3	1.2×10^8	40/92

ND=not determined

All positive clones from the second and third rounds of selection were further characterized by DNA fingerprinting to screen for the number of unique scFv, and a specificity ELISA was performed. Results are shown below in Tables 5 (BoNT/A), 6 (BoNT/B), 7 (BoNT/C) and 8 (BoNT/E), and the results summarized in Table 8.

Table 5. Fingerprint pattern and specificity ELISA signals of scFv selected on BoNT/A. Numbers in parentheses indicate the number of clones with a given fingerprint pattern.

	ELISA signal on indicated antigen							
fingerprint pattern	Clone	BoNT A	BoNT B	BoNT C	BoNT E	lysozyme	ricin	KLH
1 (27)	4E4	1.237	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
2 (55)	3A6	2.001	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
3 (1)	3B8	0.583	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
4 (1)	3E9	0.775	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
5 (1)	3E11	1.272	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
6 (2)	3F10	0.370	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
7 (4)	3D3	1.811	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
8 (9)	4A4	1.779	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
9 (1)	3E8	0.972	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
10 (1)	4B4	0.643	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

fingerprint pattern	Clone	BoNT A	BoNT B	BoNT C	BoNT E	lysozyme	ricin	KLH
11 (11)	3B12	1.372	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
12 (1)	4H4	0.427	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
13 (1)	2B10	0.964	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
14 (1)	2C7	1.763	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
15 (1)	2B11	1.384	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

Table 6. Fingerprint pattern and specificity ELISA signals of scFv selected on BoNT/B. Numbers in parentheses indicate the number of clones with a given fingerprint pattern.

	ELISA signal on indicated antigen							
fingerprint pattern	Clone	BoNT A	BoNT B	BoNT C	BoNT E	lysozyme	ricin	KLH
1 (2)	2G1	<0.05	1.087	<0.05	<0.05	<0.05	<0.05	<0.05
2 (6)	3G2	<0.05	1.179	<0.05	<0.05	<0.05	<0.05	<0.05
3 (7)	3A3	<0.05	1.827	<0.05	<0.05	<0.05	<0.05	<0.05
4 (16)	3A2	<0.05	2.099	<0.05	<0.05	<0.05	<0.05	<0.05
5 (3)	3A12	<0.05	2.112	<0.05	<0.05	<0.05	<0.05	<0.05
6 (1)	3D3	<0.05	1.079	<0.05	<0.05	<0.05	<0.05	<0.05
7 (6)	3B1	<0.05	1.865	<0.05	<0.05	<0.05	<0.05	<0.05
8 (1)	2C8	<0.05	0.904	<0.05	<0.05	<0.05	<0.05	<0.05
9 (2)	2D1	<0.05	1.567	<0.05	<0.05	<0.05	<0.05	<0.05
10 (2)	2D12	<0.05	1.026	<0.05	<0.05	<0.05	<0.05	<0.05
11 (3)	3G9	<0.05	0.970	<0.05	<0.05	<0.05	<0.05	<0.05
12 (3)	2G3	<0.05	1.638	<0.05	<0.05	<0.05	<0.05	<0.05
13 (1)	3A1	<0.05	1.309	<0.05	<0.05	<0.05	<0.05	<0.05
14 (1)	3A9	<0.05	1.518	<0.05	<0.05	<0.05	<0.05	<0.05
15 (1)	3F5	<0.05	0.994	<0.05	<0.05	<0.05	<0.05	<0.05
16 (1)	3H8	<0.05	1.031	<0.05	<0.05	<0.05	<0.05	<0.05
17 (1)	3H1	<0.05	1.288	<0.05	<0.05	<0.05	<0.05	<0.05

Table 7. Fingerprint pattern and specificity ELISA signals of scFv selected on BoNT/C. Numbers in parentheses indicate the number of clones with a given fingerprint pattern.

	ELISA signal on indicated antigen							
fingerprint pattern	Clone	BoNT A	BoNT B	BoNT C	BoNT E	lysozyme	ricin	KLH
1 (6)	2A1	<0.05	<0.05	0.31	<0.05	<0.05	<0.05	<0.05
2 (3)	2A5	<0.05	<0.05	0.42	<0.05	<0.05	<0.05	<0.05
3 (1)	3B7	<0.05	<0.05	0.27	<0.05	<0.05	<0.05	<0.05
4 (1)	2D2	<0.05	<0.05	0.39	<0.05	<0.05	<0.05	<0.05
5 (1)	3E8	<0.05	<0.05	0.62	<0.05	<0.05	<0.05	<0.05
6 (1)	3F5	<0.05	<0.05	0.44	<0.05	<0.05	<0.05	<0.05

Table 8. Fingerprint pattern and specificity ELISA signals of scFv selected on BoNT/E. Numbers in parentheses indicate the number of clones with a given fingerprint pattern.

	ELISA signal on indicated antigen							
fingerprint pattern	Clone	BoNT A	BoNT B	BoNT C	BoNT E	lysozyme	ricin	KLH
1 (4)	2A1	<0.05	<0.05	<0.05	0.440	<0.05	<0.05	<0.05
2 (5)	2A10	<0.05	<0.05	<0.05	0.405	<0.05	<0.05	<0.05
3 (1)	2C11	<0.05	<0.05	<0.05	0.634	<0.05	<0.05	<0.05

All scFv were highly specific for the serotype used for selection, with no cross reactivity observed. BoNT/A and BoNT/B appeared to more immunogenic than BoNT/E based on the number of different scFv isolated.

DNA fingerprinting is a crude estimate of the number of unique scFv present, but it is certainly possible for scFv with the same fingerprint to have different DNA sequences and specificities. This is because BstN1, the restriction enzyme used for fingerprinting, tends to cut in the V_H and V_L framework regions, rather than in the antigen binding loops (CDRs). Thus we sequenced multiple clones from each restriction pattern. The number of unique scFv for each serotype is shown below in table 9 and the sequences of the V_H and V_L genes are shown in Tables 10-14 in appendix 3.

Table 9. Summary of selection results on BoNT/A, BoNT/B, BoNT/C, and BoNT/E.

BoNT serotype used for selection	Number of unique scFv (as determined by PCR fingerprinting)
BoNT/A	23
BoNT/B	16
BoNT/C	6
BoNT/E	3

2.2B. Results from a non-immune Fab phage antibody library

In the original contract, we had proposed performing selections from a pre-existing non-immune 3.0×10^7 member scFv phage antibody library in order to produce as many scFv as possible (19). During the review interval, Griffiths et al. demonstrated that use of larger phage antibody fragment libraries yielded a greater number of binders, with better affinities (27). We therefore utilized this 7.0×10^{10} Fab phage antibody library for selections performed on BoNT/A. The library was kindly provided Dr. Greg Winter at the MRC Centre for Protein Engineering. Phage were prepared and the library selected on 50 µg/ml BoNT/A immobilized on polystyrene, and the polyclonal phage prep checked after each round of selection for binding to BoNT/A by ELISA. Binding was detected using goat polyclonal anti-M13 antibody, biotinylated mouse anti-sheep Fc, and streptavidin HRP. Results are shown in Table 15. After 4 rounds of selection the ELISA signal of polyclonal phage on BoNT A had increased from 0.05 to 1.4.

Table 15. Results of selection of a non-immune Fab library on BoNT/A.

Round of selection	ELISA signal A405 nm
1	0.05
2	0.07
3	0.7
4	1.4

Unlike phagemid vectors used for scFv phage antibody libraries, the Fab library is in a phage vector. This is necessitated by the combinatorial infection approach used to generate the library. For expression of native soluble (non-fusion) Fab, the Fab gene cassette must be subcloned. The Fab gene cassette was amplified from the polyclonal phage prep after the third and fourth rounds of selection, and cloned into the vector pUC119 mycHisXba (27). Fab was expressed from individual colonies and analyzed for binding to BoNT/A by ELISA. Binding was detected using anti-myc tag antibody and anti-mouse Fc-HRP. Results are shown in Table 16.

Table 16. Frequency of ELISA positive Fab after subcloning for expression of native Fab.

Round of selection	ELISA positive clones
3	6/92
4	15/92

15/92 Fab screened by ELISA bound BoNT/A. A single PCR fingerprint pattern was observed on screening these 14 clones, and DNA sequencing revealed a single unique Fab B10. This Fab did not bind BoNT C-fragment. Fab was expressed and purified by IMAC followed by gel filtration. The affinity of the Fab was determined by BIAcore (as described above). BoNT/A (15 μg/ml in acetate buffer pH 5.5) was immobilized to a CM5 sensor chip using EDC-NHS chemistry. The chip surface was regenerated between runs using 20 mM glycine, pH 10.5. K_d was 4.6×10^{-8} M, with a k_{on} of 3.76×10^5 $M^{-1}s^{-1}$ and a k_{off} of 1.74×10^{-2} s^{-1}.

The isolation of only a single BoNT binding Fab was unexpected, given the library size and our results with smaller scFv phage antibody libraries. We concluded that our inability to isolate a greater number of Fab resulted from 3 major library limitations: 1) difficulty in subcloning Fab genes for expression, 2) low expression levels of many Fabs resulting in inadequate quantities of material for characterization, and 3) the library was relatively unstable. These limitations are a result of creating the library in a phage vector, and the use of the cre-lox recombination system. We therefore decided that the best approach for this project was to use a very large scFv library using a phagemid vector. The goal was to produce a library at least 100 times larger than our previous 3.0 x 107 member scFv library. Creation of this library was largely funded by DAMD17-94-J-4433, however the library is summarized briefly below and the manuscript is included in appendix 2.

2.2C. Results from a non-immune scFv phage antibody library

2.2C1. Creation and validation of a 7.0×10^9 member human non-immune scFv phage antibody library

To create a very large scFv library, the approach taken was to clone the V_H and V_L library on separate replicons, combine them into an scFv gene repertoire by splicing by overlap extension, and clone the scFv gene repertoire into the phage display vector pHEN1 (Figure 7). Human peripheral blood lymphocyte and spleen RNA was primed with immunoglobulin C_κ, C_λ, and IgM primers, and 1st strand cDNA synthesized. 1st strand cDNA was used as a template for PCR amplification of the V_H, V_κ, and V_λ gene repertoires. The V_H gene repertoires were cloned into the vector pUC119Sfi-Not as NcoI-NotI fragments, to create a library of 8.0×10^8 members. The library was diverse by PCR fingerprinting. Single chain linker DNA was spliced onto the V_κ, and V_λ gene repertoires using PCR and the repertoire cloned as an XhoI-NotI fragment into the vector pHENIXscFv to create a library of 7.2×10^6 members. The V_H and V_L gene repertoires were amplified from their respective vectors and spliced together using PCR to create an scFv gene repertoire. The scFv gene repertoire was cloned as an NcoI-NotI fragment into the vector to create an scFv phage antibody library of 7.0×10^9 members. The library was diverse as determined by BstN1 fingerprinting.

To verify the quality of the library, phage were prepared and selected on 14 different protein antigens (39). The results are shown in Table 17. scFv antibodies were obtained against all antigens used for selection, with between 3 and 15 unique scFv isolated per antigen (average 8.7) (Table 17). This compares favorably to results obtained from smaller scFv libraries (1 to a few binders obtained against only 70% of antigens used for selection). Affinities of 4 anti-erbB-2 scFv and 4 anti-Botulinum scFv were measured using surface plasmon resonance in a BIAcore and found to range from 4.0×10^{-9} M to 2.2×10^{-10} M for the anti-ErbB2 scFv and 2.6×10^{-8} M to 7.15×10^{-8} M for the anti-Botulinum scFv (Table 18). scFv were highly specific for the antigen used for selection (see below for BoNT scFv) The scFv could be successfully used in a number of immunologic assays including ELISA, immunofluorescence, Western blotting, epitope mapping and immunoprecipitation. The number of binding antibodies for each antigen, and the affinities of the binding scFv are comparable to results obtained from the best phage antibody libraries (Table 19). Thus the library was established as a source of panels of human antibodies against any antigen with affinities at least equivalent to the secondary murine response.

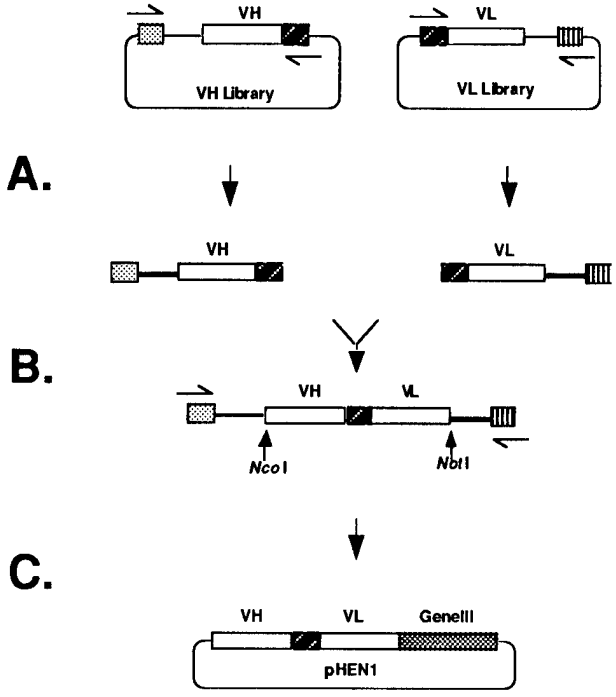


Figure 7. Method for construction of a large human scFv phage antibody library. The strategy for library construction involved optimizing the individual steps of library construction to increase both the efficiency of scFv gene assembly and to increase the efficiency of cloning assembled scFv genes. **(A).** First, mRNA from lymphocytes was used to generate V_H and V_L gene repertoires by RTPCR which were cloned into different vectors to create V_H and V_L gene libraries of 8.0×10^8 and 7.2×10^6 members respectively. The cloned V-gene libraries provided a stable and limitless source of V_H and V_L genes for scFv assembly. DNA encoding the peptide $(G_4S)_3$ was incorporated into the 5' end of the V_L library. This permitted generation of scFv genes by PCR splicing 2 DNA fragments. Previously, scFv gene repertoires were assembled from 3 separate DNA fragments consisting of V_H, V_L and linker DNA. **(B)** V_H and V_L gene repertoires were amplified from the separate libraries and assembled into an scFv gene repertoire using overlap extension PCR. The primers used to reamplify the V_H and V_L gene repertoires annealed 200 bp upstream of the 5' end of the V_H genes and 200 bp down stream of the V_L genes. These long overhangs ensured efficient restriction enzyme digestion.**(C.)** The scFv gene repertoire was digested with *Nco*I and *Not*I and cloned into the plasmid pHEN1 as fusions with the M13 gene III coat protein gene (▩) for phage-display.

Table 17. Results of phage antibody library selections. For each antigen (column 1), the number and the percentage of positive clones selected (column 2) and the number of different antibodies isolated (column 3) is indicated.

Protein antigen used for selection	Percentage (number) of ELISA positive clones	Number of different antibodies isolated
FGF Receptor ECD	69 (18/26)	15
BMP Receptor Type I ECD	50 (12/24)	12
Activin Receptor Type I ECD	66 (16/24)	7
Activin Receptor Type II ECD	66 (16/24)	4
Erb-B2 ECD	91 (31/34)	14
VEGF	50 (48/96)	6
BoNT/A	28 (26/92)	14
BoNT-A C-fragment	95 (87/92)	10
BoNT/B	10 (9/92)	5
BoNT/C	12 (11/92)	5
BoNT/E	9 (8/92)	3
Bungarotoxin	67 (64/96)	15
Cytochrome b5	55 (53/96)	5
Chlamydia trachomatis EB	66 (63/96)	7

Table 18. Affinities and binding kinetics of anti-BoNT/A C-fragment and anti-Erb-B2 scFv. Association (k_{on}) and dissociation (k_{off}) rate constants for purified scFvs were measured using surface plasmon resonance (BIAcore) and K_d calculated as (k_{off}/k_{on}).

Specificity and clone	K_d (x 10^{-9}M)	k_{on} (x $10^5 M^{-1}s^{-1}$)	k_{off} (x $10^{-3}s^{-1}$)
ErbB-2 B7A	0.22	4.42	0.1
ErbB-2 G11D	0.48	2.19	0.11
ErbB-2 A11A	0.49	3.69	0.18
ErbB-2 F5A	4.03	1.62	0.65
BoNT-A 2A9	26.1	0.25	0.66
BoNT-A 2H6	38.6	2.2	8.5
BoNT-A 3F6	66.0	4.7	30.9
BoNT-A 2B6	71.5	1.1	7.8

Table 19. Comparison of protein binding antibodies selected from non-immune phage-display antibody libraries. * For library type, N = V-gene repertoires obtained from V-genes rearranged in vivo; SS = semi-synthetic V-genes constructed from cloned V-gene segments and synthetic oligonucleotides encoding V_H CDR3. ND = not determined.

Library	Library size and type*	Number of protein antigens studied	Average number of antibodies per protein antigen	Number of affinities measured	Range of affinities for protein antigens K_d (x 10^{-9}M)
Marks *et al* (19)	3.0×10^7 (scFv, N)	2	2.5	1	100-2000
Nissim *et al* (41)	1.0×10^8 (scFv, SS)	15	2.6	ND	ND
deKruif *et al* (42)	3.6×10^8 (scFv, SS)	12	1.9	3	100 - 2500
Griffiths *et al* (27)	6.5×10^{10} (Fab, SS)	30	4.8	3	7 - 58
Vaughan *et al* (28)	1.4×10^{10} (scFv, N)	3	7.0	3	4.2 - 8.0
Sheets *et al* (this work)	6.7×10^9 (scFv, N)	14	8.7	8	0.22 - 71.5

2.2C2. Selection and characterization of BoNT binding scFv from a non-immune library

scFv binding BoNT/A, BoNT/B, BoNT/C, BoNT/E, and BoNT/A C-fragment were selected and characterized as described above in section 3.1 above. Results are shown below in Tables 20 and 21.

Table 20. Results of selection of a non-immune scFv phage antibody library on BoNT/A, BoNT/B, BoNT/C, and BoNT/E.

Antigen used for selection	Round of selection	Eluted phage titre	ELISA positive scFv
BoNT type A	1	5.0×10^5	0/92
	2	3.0×10^5	8/92
	3	5.0×10^7	10/92
BoNT type B	1	1.0×10^4	0/92
	2	4.9×10^4	7/92
	3	2.4×10^8	9/92
BoNT type C	1	1.0×10^4	0/92
	2	3.3×10^5	10/92
	3	3.3×10^8	11/92
BoNT type E	1	1.0×10^4	0/92
	2	1.8×10^5	1/92
	3	4.0×10^8	4/92

Table 21. Results of selection of a non-immune scFv phage antibody library on BoNT/A C-fragment.

Round of selection	Eluted phage titre	BoNT A C-fragment ELISA positive scFv	BoNT A ELISA positive scFv
1	4.0×10^4	0/92	ND
2	2.0×10^6	13/92	10/92
3	1.5×10^9	14/92	11/92

All positive clones from the second and third rounds of selection were further characterized by DNA fingerprinting to screen for the number of unique scFv, and a specificity ELISA was performed. Results are shown below in Tables 22 (BoNT/A), 23 (BoNT/B), 24 (BoNT/C), 25 (BoNT/E), and 26 (BoNT/A C-fragment).

Table 22. Fingerprint pattern and specificity ELISA signals of scFv selected on BoNT/A. Numbers in parentheses indicate the number of clones with a given fingerprint pattern.

	ELISA signal on indicated antigen							
fingerprint pattern	Clone	BoNT A	BoNT B	BoNT C	BoNT E	lysozyme	ricin	KLH
1 (2)	2D8	0.323	<0.315	<0.05	<0.05	<0.05	<0.05	<0.05
2 (2)	3D1	0.795	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
3 (2)	4C10	0.697	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
4 (1)	3F6	0.522	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
5 (2)	3H3	0.701	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

Table 23. Fingerprint pattern and specificity ELISA signals of scFv selected on BoNT/B. Numbers in parentheses indicate the number of clones with a given fingerprint pattern.

	ELISA signal on indicated antigen							
fingerprint pattern	Clone	BoNT A	BoNT B	BoNT C	BoNT E	lysozyme	ricin	KLH
1 (6)	3D1	<0.05	0.788	<0.05	<0.05	<0.05	<0.05	<0.05
2 (4)	3D7	<0.05	0.834	0.218	<0.05	<0.05	<0.05	<0.05
3 (1)	2A5	0.327	0.635	<0.05	<0.05	<0.05	<0.05	<0.05
4 (1)	3B6	<0.05	0.532	<0.05	<0.05	<0.05	<0.05	<0.05
5 (1)	2A12	<0.05	0.540	<0.05	<0.05	<0.05	<0.05	<0.05
6 (4)	3A12	<0.05	0.782	<0.05	<0.05	<0.05	<0.05	<0.05

Table 24. Fingerprint pattern and specificity ELISA signals of scFv selected on BoNT/C. Numbers in parentheses indicate the number of clones with a given fingerprint pattern.

	ELISA signal on indicated antigen							
fingerprint pattern	Clone	BoNT A	BoNT B	BoNT C	BoNT E	lysozyme	ricin	KLH
1 (15)	3C4	<0.05	<0.05	1.349	<0.05	<0.05	<0.05	<0.05
2 (5)	2B12	<0.05	<0.05	0.710	<0.05	<0.05	<0.05	<0.05
3 (1)	2F10	<0.05	<0.05	0.410	<0.05	<0.05	<0.05	<0.05
4 (1)	3B6	<0.05	<0.05	0.629	<0.05	<0.05	<0.05	<0.05
5 (1)	2A9	<0.05	<0.05	0.352	<0.05	<0.05	<0.05	<0.05
6 (4)	3D5	<0.05	0.440	0.375	<0.05	<0.05	<0.05	<0.05

Table 25. Fingerprint pattern and specificity ELISA signals of scFv selected on BoNT/E. Numbers in parentheses indicate the number of clones with a given fingerprint pattern.

	ELISA signal on indicated antigen							
fingerprint pattern	Clone	BoNT A	BoNT B	BoNT C	BoNT E	lysozyme	ricin	KLH
1 (7)	3C8	<0.05	<0.05	<0.05	0.635	<0.05	<0.05	<0.05
2 (1)	2D7	<0.05	<0.05	<0.05	0.325	<0.05	<0.05	<0.05

Table 26. Fingerprint pattern and specificity ELISA signals of scFv selected on BoNT/A C-fragment.

		ELISA signal on indicated antigen				
fingerprint pattern	Clone	BoNT A C-fragment	BoNT A	BSA	KLH	lysozyme
1 (2)	2B10	0.872	0.619	<0.05	<0.05	<0.05
2 (2)	2A9	1.658	0.661	<0.05	<0.05	<0.05
3 (2)	2B1	0.658	0.637	<0.05	<0.05	<0.05
4 (2)	3B7	0.780	0.449	<0.05	<0.05	<0.05
5 (1)	2B6	0.621	0.335	<0.05	<0.05	<0.05
6 (2)	3B10	0.695	0.598	<0.05	<0.05	<0.05
7 (2)	2H6	0.778	0.977	<0.05	<0.05	<0.05
8 (1)	3C2	0.798	0.363	<0.05	<0.05	<0.05

The V_H and V_L genes of at least 2 clones of each DNA fingerprint pattern were sequenced and the partial sequences are shown in Tables 27-31 in appendix 3. The number of unique scFv identified by DNA sequencing is summarized in Table 32.

Table 32. Summary of selection results on BoNT/A, BoNT/B, BoNT/C, and BoNT/E.

BoNT type used for selection	Number of unique scFv (as determined by DNA sequencing)
BoNT/A	5
BoNT/B	5
BoNT/C	5
BoNT/E	3
BoNT/A C-Fragment	10

2.2D. Further characterization of BoNT/A binding scFv from immune and non-immune human phage antibody libraries

Since our contract is focused on producing antibodies which neutralize BoNT/A, we further characterized the 23 immune and 15 non-immune scFv which bound BoNT/A. The domain specificity of each scFv was determined by specificity ELISA performed as described above, but using BoNT/A, BoNT/A C-fragment (Ophidian) or BoNT translocation domain (H_N) coated at 50 µg/ml. To produce translocation domain, the gene encoding the translocation domain was amplified by PCR and cloned into the pET vector (Novagen) with expression under control of the T7 promoter. This work was performed in the Marks laboratory. Recombinant translocation domain was expressed and purified by immobilized metal affinity chromatography (IMAC) in the laboratory of Dr. Ray Stevens at U.C. Berkeley. The results of the domain specificity are shown below in Table 33.

Table 33. Domain specificity of human BoNT/A binding scFv selected from phage antibody libraries.

	Number of unique scFv		
scFv Specificity	Humans immunized with toxoid	Non-immune humans	Total scFv
BoNT/A H_C	6	10	16
BoNT/A H_N	4	2	6
BoNT/A light chain	16	3	19

For subsequent characterization of scFv for neutralization capacity, we focused on those scFv which bound BoNT/A H_C since polyclonal antibodies to mice immunized with this domain have been shown to be protected against toxin (33). Since the number of scFv for *in vitro* neutralization studies was large (16 scFv) we sought to reduce the number by first epitope mapping and ranking the k_{off} of each anti-BoNT/A scFv. We would then study the highest affinity scFv to each epitope to identify those with neutralizing capacity. Epitope mapping and

affinity measurements were performed by BIAcore exactly as described in reference (37). The results are summarized below in Table 34. Clone 2B1 could not be epitope mapped due to its low expression levels. From the immune library, scFv recognized two different epitope clusters. scFv binding one of these clusters (cluster 1) neutralized BoNT/A *in vitro*. From the non-immune library, many more epitope clusters were recognized. No non-immune scFv, however, exhibited *in vitro* neutralization. scFv with homologous V_H CDR3s recognized the same epitopes (Table 35).

Table 34. Affinities, binding kinetics, and *in vitro* toxin neutralization results of scFv selected from phage antibody libraries.

Clone	Cluster	K_d † (M)	k_{on} (10^5 M^{-1} s^{-1})	k_{off} (10^{-3} s^{-1})	Paralysis Time¥
Immune Library					
3D12§	I	3.69×10^{-8}	0.13	0.50	85 ± 5.0[b]
2A2	I	ND			
2B10	I	ND			
3F10§	II	7.80×10^{-9}	0.80	0.62	55± 5.0[d]
3B6	II	ND			
2B1	ND	ND			
Non-Immune Library					
2B10*	III	1.29×10^{-7}	5.57	71.6	62.3± 6.7[d]
2A2	III	ND			
2E6*	IV	1.93×10^{-7}	1.19	23.0	60.9 ± 8.2[d]
2H6*	V	3.86×10^{-8}	2.20	8.50	63.0± 5.0[d]
2B1*	VI	1.07×10^{-7}	0.83	8.88	58.4± 4.0[d]
2A9*	VII	2.61×10^{-8}	0.25	0.66	71.0± 3.0[d]
2B6*	VIII	7.15×10^{-8}	1.09	7.80	61.9± 5.0[d]
3D1*	IX	4.60×10^{-7}	1.31	60.3	58.3± 3.8[d]
3F6§	X	6.60×10^{-8}	4.69	30.9	60.4± 3.6[d]
3C2*	XI	3.90×10^{-8}	2.10	82.0	61.9± 4.8[d]

† k_{on} and k_{off} were measured by Biacoree and K_d calculated as k_{off}/k_{on} ¥ Time (min.) to 50% twitch reduction in mouse hemidiaphragm assay using 20 nM scFv + 20 pM BoNT/A, compared to time for BoNT/A alone. § Library selected on BoNT/A,. * Library selected on BoNT/A H_C., [a] $p<0.05$, [b] $p<0.01$ compared to BoNT/A, [c] $p<0.01$ compared to BoNT/A, [d] not significant ND = not determined

Table 35. CDR3-Sequences and affinities for human scFv antibodies isolated from immune and non-immune libraries, selected on BoNT/A and BoNT/A H_C.

Clone	Heavy Chain Family	Segment	Difference from germline	V_H CDR 3	Light Chain Family	Segment	Difference from germline	V_L CDR 3
Non-immune Library								
2A2*	VH3	DP46	0	DLDYGGNAGYFDL	Vλ3	DPL16	10	HSRDSSVTNLD
2B10*	VH3	DP46	0	DLDYGGNAGYFDL	Vλ3	DPL16	4	NSRDSSGNHQV
2E6*	VH3	DP46	0	DYTANYYYYGMDV	Vλ2	DPL12	14	NSRDSSGVV
2H6*	VH3	DP47	6	ALQSDSPYFD	Vλ3	DPL16	7	NSRDSSGNHVV
3C2*	VH3	DP46	2	DLAIFAGNDY	Vλ3	DPL16	9	KSRDSRGNHLAL
2B1*	VH3	DP46	0	NGDPEAFDY	Vκ1	L1	11	LQDYNGWT
2A9*	VH3	DP54	5	GRGVN	Vκ1	L12A	6	QQANSFPRT
2B6*	VH3	DP47	3	VGVDRWYPADY	Vκ1	L12A	5	QQYHTISRT
3D1*	VH3	DP47	7	DLGYGSGTSSYYLDY	Vλ3	DPL16	5	NSRDSSGNHVV
3F6§	VH3	DP47	2	DLLDGSGAYFDY	Vλ3	DPL16	3	NSRDSSGNHVV

Clone	Heavy Chain Family	Segment	Difference from germline	V_H CDR 3	Light Chain Family	Segment	Difference from germline	V_L CDR 3
Immune Library								
3A6§	VH3	DP50	18	EPDWLLWGDRGALDV	Vκ1	L12	8	QHYNTYPYT
3D12§	VH3	DP50	13	EPDWLLWGDRGALDV	Vκ1	L12	10	QHYNTYPYT
2A1*	VH3	DP50	14	EPDWLLWGDRGALDV	Vκ1	L12	4	QHYNTYPYT
3B8§	VH1	V1-2	10	LATYYYFGLDV	Vκ1	DPK7	12	QQYNSYVYT
3F10§	VH1	V1-2	10	LATYYYFGLDV	Vκ1	DPK8	10	QQLNSYPLT
2B11§	VH1	DP10	11	GPWELVGYFDS	Vκ1	L12	11	QQLLSYPLT

§ Library selected on BoNT/A, *Library selected on BoNT/A H_C. Human germline V_H, V_k and V_l segments have been assigned as detailed in the V-BASE database (MRC Centre for Protein Engineering, Cambridge, UK). Listed clones, with identical V_H or V_L CDR 3 regions, showed different CDR 1, CDR 2 and Framework regions, as indicated by their differences from the germline genes

2.2D. Isolation and characterization of murine scFv from a mouse immunized with recombinant BoNT/A C-fragment

The methodology for the experiments described below have largely been described above and are also outlined in detail in the manuscript in appendix 1 (37).

2.2D1. Murine phage antibody library construction

Two phage antibody libraries were constructed from the V_H and V_κ genes of immunized mice. For library 1, a mouse was immunized twice with BoNT/A H_C and challenged 2 weeks after the second immunization with 100,000 LD_{50}s of BoNT/A. The mouse survived the BoNT/A challenge, and 1 week later was sacrificed. The spleen was removed immediately after sacrifice and total RNA prepared. For library construction, IgG heavy chain and kappa light chain mRNA were specifically primed and 1st strand cDNA synthesized. V_H and V_κ gene repertoires were amplified using PCR and V_H, J_H, V_κ and J_κ primers provided in the recombinant phage antibody system. The V_H and V_K gene repertoires were randomly spliced together to create a scFv gene repertoire using synthetic DNA encoding the 15 amino acid peptide linker $(G_4S)_3$. Each scFv gene repertoire was separately cloned into the phage display vector pCANTAB5E (Pharmacia). After transformation, a library of 2.1×10^6 members was obtained. 90% of clones had an insert of the appropriate size for a scFv gene, as determined by PCR screening and the cloned scFv genes were diverse as determined by PCR fingerprinting. DNA sequencing of 10 unselected clones from library 1 revealed that all V_H genes were derived from the murine V_H2 family and all V_κ genes were derived from the murine $V_\kappa4$ and $V_\kappa6$ families. Based on this observed V-gene bias, family specific V_H and V_κ primers were designed along with J_H and J_κ gene segment specific primers (Table 1 in appendix 1). These primers were then used to construct a second phage antibody library. For library 2, a mouse was immunized three times with BoNT/A H_C and sacrificed 2 weeks after the third immunization. The mouse was not challenged with BoNT/A prior to spleen harvest as this led to the production of non H_C binding antibodies (see results of selection, below). The spleen was harvested and a phage antibody library constructed as described above except that V_H, J_H, V_κ, and J_κ specific primers were used. After transformation, a library of 1.0×10^6 members was obtained. 95% of clones had an insert of the appropriate size for a scFv gene, as determined by PCR screening and the cloned scFv genes were diverse as determined by PCR fingerprinting (data not shown). DNA sequencing of 10 unselected clones from library 2 revealed greater diversity than from library 1; V_H genes were derived from the V_H1, V_H2, and V_H3 families and V_κ genes were derived from the $V_\kappa2$, $V_\kappa3$, $V_\kappa4$, and $V_\kappa6$ families.

2.2D2. Selection and initial characterization of phage antibodies

To isolate BoNT/A binding phage antibodies, phage were rescued from the library and selected on either purified BoNT/A or BoNT/A H_C. Selections were performed on the holotoxin in addition to H_C since it was unclear the extent to which the recombinant toxin H_C would mimic the conformation of the H_C in the holotoxin. Selection for BoNT/A and BoNT/A

H_C binders was performed on antigen adsorbed to polystyrene. In addition H_C binding phage were selected in solution on biotinylated H_C, with capture on streptavidin magnetic beads (library 1) or on hexahistidine tagged H_C (library 2), with capture on Ni^{+2}-NTA agarose. Selections in solution were utilized based on our previous observation that selection on protein adsorbed to polystyrene could yield phage antibodies which did not recognize native protein (40). Selection in solution was not performed on the holotoxin due to our inability to successfully biotinylate the toxin without destroying immunoreactivity.

After two to three rounds of selection, at least 67% of scFv analyzed bound the antigen used for selection (Table 36). The number of unique scFv was determined by DNA fingerprinting followed by DNA sequencing, and the specificity of each scFv was determined by ELISA on pure BoNT/A and recombinant BoNT/A H_C and H_N. scFv binding BoNT/A but not binding H_C or H_N were presumed to bind the light chain (catalytic domain). A total of 33 unique scFv were isolated from mice immunized with H_C and challenged with BoNT/A (library 1, Table 37). When library 1 was selected on holotoxin, 25 unique scFv were identified. Only 2 of these scFv, however, bound H_C, with the majority (21) binding the light chain and two binding H_N. Selection of library 1 on H_C yielded an additional 8 unique scFv (Tables 36 and 37). Overall, however, only 50% of scFv selected on H_C also bound holotoxin. This result suggests that a significant portion of the H_C surface may be inaccessible in the holotoxin. Alternatively, scFv could be binding H_C conformations that do not exist in the holotoxin. From mice immunized with H_C only (library 2) all scFv selected on holotoxin also bound H_C. As with library 1, however, only 50% of scFv selected on H_C bound holotoxin. In all, 18 unique H_C binding scFv were isolated from library 2, resulting in a total of 28 unique H_C binding scFv (Tables 37 (see below) and Table 38 (in appendix 3). scFv of identical or related sequences were isolated on both H_C immobilized on polystyrene and in solution. Thus in the case of H_C, the method of selection was not important.

Table 36. Frequency of binding clones from immune murine phage antibody libraries

Antigen used for selection	Frequency of ELISA positive clones[a]		
	Round of selection		
	1	2	3
A. Library 1[b]			
BoNT/A: immunotube[c]	20/184	124/184	ND
BoNT/A H_C: immunotube	7/92	86/92	88/92
BoNT/A H_C: biotinylated[d]	7/90	90/90	90/90
	14/48	48/48	ND
B. Library 2[e]			
BoNT/A: immunotube	ND	81/92	ND
BoNT/A H_C: immunotube	ND	ND	76/92
BoNT/A H_C: Ni^{++}-NTA[f]	ND	ND	67/92

[a] For selections on BoNT/A and BoNT/A H_C, ELISA done on immobilized BoNT/A and BoNT/A H_C, respectively. [b] library 1 derived from a mouse immunized twice with BoNT/A H_C and once with BoNT/A. [c] Immunotube selections performed with the antigen absorbed onto immunotubes.

[d] Biotinylated selections performed in solution with capture on streptavidin magnetic beads. [e] library 2 derived from a mouse immunized three times with BoNT/A H_C. [f] Ni^{++}-NTA selections performed in solution with capture on Ni^{++}-NTA agarose. ND, data not determined from selection performed.

Table 37. Specificity of BoNT binding scFv selected from phage antibody libraries

scFv Specificity	Number of unique scFv	
	library 1	library 2
BoNT/A H_C	10	18
BoNT/A H_N	2	0
BoNT/A light chain	21	0
Total	33	18

2.2D3. Epitope mapping of murine scFv

All 28 unique H_C binding scFv were epitope mapped using surface plasmon resonance in a BIAcore. Mapping of the 28 scFv yielded 4 non-overlapping epitopes recognized on H_C (Table 38). scFv recognizing only epitopes 1 and 2 were obtained from, library 1, whereas scFv recognizing all 4 epitopes were obtained from library 2. Many of the scFv recognizing the same epitope (C1 and S25, C9 and C15; 1E8 and 1G7; 1B6 and 1C9; C25 and C39; 2G5, 3C3, 3F4, and 3H4; 1A1 and 1F1; 1B3 and 1C6; 1G5 and 1H6; 1F3 and 2E8) had V_H domains derived from the same V-D-J rearrangement, as evidenced by the high homology of the V_H CDR3 and V_H-gene segment. These scFv differ only by substitutions introduced by somatic hypermutation or PCR error. For epitopes 1 and 2, most or all of the scFv recognizing the same epitope are derived from the same or very similar V_H gene segments but differ significantly with respect to V_H CDR3 length and sequence (5/9 scFv, epitope 1; 8/8 scFv, epitope 2) (Table 38). These include scFv derived from different mice. Given the great degree of diversity in V_H CDR2 sequences in the primary repertoire (43), specific V_H gene segments may have evolved for their ability to form binding sites capable of recognizing specific pathogenic antigenic shapes. In contrast, greater structural variation appears to occur in the rearranged V_K genes. For example, three different germline genes and CDR1 main chain conformations (44) are observed for epitope 2, where all the V_H genes are derived from the same germline gene. Such 'promiscuity' in chain pairings has been previously reported (23).

2.2D4. Affinity, binding kinetics, and *in vitro* toxin neutralization

Affinity, binding kinetics, and *in vitro* toxin neutralization were determined on one representative scFv binding to each epitope. For each epitope, the scFv chosen for further study had the best combination of expression level and slowest k_{off}, as determined during epitope mapping studies. K_d for the four scFv studied ranged between 7.3×10^{-8} M and 1.1×10^{-9} M (Table 39), values comparable to those reported for monoclonal IgG produced from hybridomas (38). C25 has the highest affinity ($K_d = 1.1 \times 10^{-9}$ M) reported for an anti-botulinum toxin antibody. k_{on} differed over 84 fold and k_{off} differed over 33 fold between scFv (Table 39). *In vitro* toxin neutralization was determined using a mouse hemidiaphragm preparation and measuring the time to 50% twitch tension reduction for BoNT/A alone and in the presence of 2.0×10^{-8} M scFv. Values are reported in time to 50% twitch reduction. scFv binding to epitope 1 (S25) and epitope 2 (C25) significantly prolonged the time to neuroparalysis 1.5 fold (52%) and 2.7 fold (270%) respectively. In contrast, scFv binding to epitopes 3 and 4 had no significant effect on the time to neuroparalysis. A mixture of S25 and C25 had a significant additive effect on the time to neuroparalysis, with the time to 50% twitch reduction increasing to 3.9 fold (390%).

Table 39. Affinities, binding kinetics, and *in vitro* toxin neutralization results of scFv selected from phage antibody libraries

scFv clone	Epitope	K_d[a] (M)	k_{on} ($\times 10^4 M^{-1} s^{-1}$)	k_{off} ($\times 10^{-3} s^{-1}$)	Paralysis Time[b]
S25	1	7.3×10^{-8}	1.1	0.82	85 ± 10[c]
C25	2	1.1×10^{-9}	30	0.33	151 ± 12[c]
C39	2	2.3×10^{-9}	14	0.32	139 ± 8.9[c]
1C6	3	2.0×10^{-8}	13	2.5	63 ± 3.3
1F3	4	1.2×10^{-8}	92	11	52 ± 1.4
C25 + S25 Combination					218 ± 22[c, d]
BoNT/A pure toxin (control)					56 ± 3.8

[a] k_{on} and k_{off} were measured by surface plasmon resonance and K_d calculated as k_{off}/k_{on}. [b] Time (min.) to 50% twitch reduction in mouse hemidiaphragm assay using 20 nM scFv + 20 pM BoNT/A, compared to time for BoNT/A alone. For C25 + S25 combination, 20 nM scFv each was used. Each value is the mean ± SEM of at least three observations. [c] $p < 0.01$ compared to BoNT/A. [d] $p < 0.05$ compared to C25

3. Conclusions

3.1 Phage libraries are a powerful approach to generating monoclonal antibodies to BoNT

Using phage display and immune and non-immune phage antibody libraries, a total of 130 unique scFv were generated. This included 51 scFv from immunized mice, 51 scFv from immunized humans and 28 scFv from a non-immune antibody library.

3.2. Non-immune phage antibody libraries are a rapid means of generating serotype specific antibodies

We have generated a non-immune phage antibody library that can generate a panel of antibodies to any protein antigen. Once a non-immune library is generated, antibodies can be selected in less than 5 days giving a polyclonal mixture that can specifically recognize antigen. Cloning out the monoclonals takes several more days. Libraries such as these can be extremely useful tools for developing detecting to new or novel biowarfare agents. The non-immune approach makes it possible to make specific antibodies faster than any other technique. In the case of BoNT, no neutralizing antibodies were obtained from the non-immune library, but that may not be the case for all toxins.

3.3 The BoNT/A H_C is a more potent immunogen for generating neutralizing antibodies

The most potent neutralzing antibodies were obtained from mice immunized with the BoNT/A H_C fragment which represents the cellular binding domain. Neutralizing antibodies recognizing two non-overlapping epitopes were obtained with this immunogen. This is not surprising since the most effective route for antibodies to neutralize toxin is to prevent binding to the cellular receptor. In contrast, a relatively small percentage of antibodies from humans immunized with polyvalent toxoid recognized BoNT/A H_C. Neutralizing antibodies recognizing only 1 epitope were obtained with this immunogen.

3.4 There appear to be two neutralizing epitopes on BoNT/A H_C

Results from both the murine and human libraries indicate an additivity for either *in vitro* or *in vivo* toxin neutralization when scFv or scFv-Fc fusions recognizing two non-overlapping epitopes on H_C are co-administered. This could represent either a broad receptor binding surface on the toxin (for one cellular receptor) or the presence of two cellular receptors for toxin. We need to confirm the importance of blocking both receptors by repeating these studies using a combination of one antibody that recognizes a neutralizing epitope and one that does not.

3.5. BoNT/A neutralizing antibodies have been generated

Multiple neutralizing murine antibodies recognizing two different epitopes and multiple human antibodies recognizing a single epitope have been produced. None of these antibodies by themselves provides high titer toxin neutralization *in vivo*. The most potent of these are murine antibodies recognizing one of these epitopes. The failure to get additional human neutralizing antibodies is likely to be due to the nature of the toxoid immunogen and the relatively small number of circulating B-lymphocytes in human blood (the source of Ig genes for library construction. It is likely that the potency of these existing antibodies can be increased by increasing the serum half life. This can be accomplished by removing the glycosylation site for the Pichia produced scFv-Fc fusions or expressing these fusions or complete IgG in mammalian cells. It should also be possible to increase potency by increasing antibody affinity.

4. Future Work

We are continuing this work under a new contract. We plan on accomplishing the listed objectives with the overall goal of generating neutralizing antibodies to the botulinum neurotoxins.

4.1 Complete *in vivo* evaluation of 3D12 scFv-Fc fusion (a human antibody that neutralizes toxin in vitro)

4.2 Remove glycosylation site from human Fc to determine effect of increased serum concentration of scFv-Fc fusion on toxin neutralization.

4.3 Determine the role of affinity on toxin neutralization by increasing affinity of the most potent in vivo neutralizing scFv-Fc fusion (either S25, C25 or 3D12 depending on the results of *in vivo* studies.

4.4 Construct new libraries and antibodies from Xenomice immunized with BoNT/A, BoNT/B and BoNT/E HC. The Xenomice are transgenic for the human Ig locus and thus generate human antibodies. The fact that we are working with mice means that we can use the preferred immunogen for generating neutralizing antibodies (the H_C) and we can harvest spleens (the best source for Ig genes). This work will be done in collaboration with Abgenix.

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Appendix One

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Molecular Characterization of Murine Humoral Immune Response to Botulinum Neurotoxin Type A Binding Domain as Assessed by Using Phage Antibody Libraries

PETER AMERSDORFER,[1] CINDY WONG,[1] STEVEN CHEN,[1] THERESA SMITH,[2] SHARAD DESHPANDE,[3] ROBERT SHERIDAN,[3] RICARDA FINNERN,[1]† AND JAMES D. MARKS[1]*

Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, San Francisco General Hospital, San Francisco, California 94110[1]; Toxicology Division, U.S. Army Medical Research Institute for Infectious Diseases, Frederick, Maryland 21702[2]; and Neurotoxicology Branch, Pathophysiology Division, U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland 21010[3]

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To produce antibodies capable of neutralizing botulinum neurotoxin type A (BoNT/A), the murine humoral immune response to BoNT/A binding domain (H_C) was characterized at the molecular level by using phage antibody libraries. Mice were immunized with BoNT/A H_C, the spleens were harvested, and single-chain Fv (scFv) phage antibody libraries were constructed from the immunoglobulin heavy and light chain variable region genes. Phage expressing BoNT/A binding scFv were isolated by selection on immobilized BoNT/A and BoNT/A H_C. Twenty-eight unique BoNT/A H_C binding scFv were identified by enzyme-linked immunosorbent assay and DNA sequencing. Epitope mapping using surface plasmon resonance in a BIAcore revealed that the 28 scFv bound to only 4 nonoverlapping epitopes with equilibrium constants (K_d) ranging from 7.3×10^{-8} to 1.1×10^{-9} M. In a mouse hemidiaphragm assay, scFv binding epitopes 1 and 2 significantly prolonged the time to neuroparalysis, 1.5- and 2.7-fold, respectively, compared to toxin control. scFv binding to epitopes 3 and 4 showed no protection against neuroparalysis. A combination of scFv binding epitopes 1 and 2 had an additive effect on time to neuroparalysis, which increased to 3.9-fold compared to the control. The results suggest that there are two "productive" receptor binding sites on H_C which lead to toxin internalization and toxicity. Blockade of these two epitopes with monoclonal antibodies may provide effective immunoprophylaxis or therapy against BoNT/A intoxication.

Botulism is a life-threatening flaccid paralysis caused by a neurotoxin produced by the anaerobic bacterium *Clostridium botulinum*. The disease typically results from ingestion of preformed toxin present in contaminated food (15) or from toxin produced in vivo from infected wounds (50) or in the intestines of infants (2) (or occasionally adults). In severe cases, patients require prolonged hospitalization in an intensive-care unit and mechanical ventilation. Specific therapy consists of administration of botulism antitoxin trivalent (equine) (48); however, this product has a high incidence of side effects, including serum sickness and anaphylaxis (5). To avoid these side effects, human BIG has been produced from immunized volunteers and its efficacy is being determined in a prospective randomized trial in infants with botulism (1). While theoretically nontoxic, human BIG also has limitations, largely related to production issues. These include potential transmission of blood-borne infectious diseases, variability in potency and specificity between lots, and the need to immunize humans. The latter issue has taken on increased importance with the use of BoNTs for the treatment of a range of neuromuscular diseases (28, 41). Immunization of volunteers for production of BIG would deprive them of subsequent botulinum therapy.

As an alternative to immune globulin, neutralizing monoclonal antibodies with defined potency and specificity could be produced in unlimited quantities. To date, however, no efficacious neutralizing antibotulinum monoclonal antibodies have been produced (38). Potential explanations for this failure include the following: (i) a neutralizing epitope(s) is less immunogenic than other epitopes; (ii) too few unique monoclonal antibodies have been studied; (iii) a toxoid immunogen (formaldehyde-inactivated crude toxin) which poorly mimics the conformation of the neutralizing epitope(s) has been used; and (iv) multiple epitopes must be blocked in order to achieve efficient neutralization (32). To address these issues, and to generate neutralizing antibodies to BoNT/A, we have produced and characterized a large panel of monoclonal antibodies from immunized mice. To generate antibodies capable of preventing the binding of toxin to its cellular receptor(s), mice were immunized with BoNT/A H_C (33). This domain contains the region(s) thought to bind to presynaptic neuronal receptors, the first requisite step for intoxication, and results in protective immunity when used as an immunogen (11, 33). To produce and characterize the greatest number of monoclonal antibodies possible, we used phage display (10, 37; reviewed in reference 34). Murine V_H and V_κ genes were used to construct libraries of millions of recombinant scFv, which were displayed on the surface of filamentous bacteriophage (Fig. 1). Phage displaying antibodies binding BoNT/A were isolated by affinity chromatography. Here we report the molecular and biophysical characterization of these antibodies, including specificity, affinity, epitopes recognized, and in vitro neutralization capacity.

* Corresponding author. Mailing address: University of California, San Francisco, San Francisco General Hospital, 1001 Potrero Ave., Rm. 3C-38, San Francisco, CA 94110. Phone: (415) 206-3256. Fax: (415) 206-3253. E-mail: jim_marks@quickmail.ucsf.edu.

† Present address: Institut de Biochemie, Université de Lausanne, 1066 Epalinges, Switzerland.

MATERIALS AND METHODS

Abbreviations used. AMP, ampicillin; BIG, botulinum immune globulin; BoNT, botulinum neurotoxin; BoNT/A, BoNT type A; CDR, complementarity-

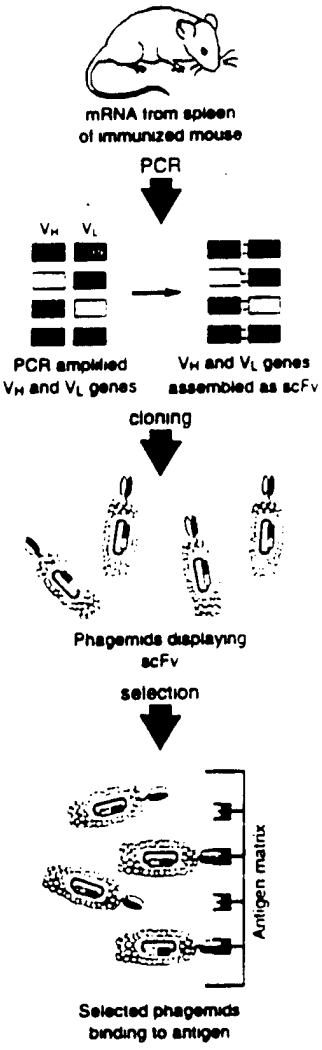

FIG. 1. Strategy for in vitro antibody production using phage display libraries. mRNA is prepared from splenocytes, first-strand cDNA is prepared, and antibody V_H and V_L genes are amplified by PCR. V_H and V_L genes are spliced together randomly by using PCR to create a repertoire of scFv genes. The scFv gene repertoire is cloned into a phagemid vector in frame with a gene (gIII) encoding a phagemid minor coat protein (pIII). Each phage in the resulting phage antibody library expresses an scFv-pIII fusion protein on its surface and contains the gene encoding the scFv inside. Phage antibodies binding a specific antigen can be separated from nonbinding phage antibodies by affinity chromatography on immobilized antigen. A single round of selection increases the number of antigen-binding phage antibodies by a factor ranging from 20 to 10,000, depending on the affinity of the antibody. Eluted phage antibodies are used to infect *E. coli*, which then produces more phage antibodies for the next round of selection. Repeated rounds of selection make it possible to isolate antigen-binding phage antibodies that were originally present at frequencies of less than one in a billion.

determining region; ELISA, enzyme-linked immunosorbent assay; GLU, glucose; HBS, HEPES-buffered saline (10 mM HEPES, 150 mM NaCl [pH 7.4]); H_C, C-terminal domain of BoNT heavy chain (binding domain); H_N, N-terminal domain of BoNT heavy chain (translocation domain); IgG, immunoglobulin G; IMAC, immobilized-metal affinity chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; KAN, kanamycin; K_d, equilibrium constant; k_{off}, dissociation rate constant; k_{on}, association rate constant; MPBS, skim milk powder in PBS; NTA, nitrilotriacetic acid; PBS, phosphate-buffered saline (25 mM NaH_2PO_4, 125 mM NaCl [pH 7.0]); RU, resonance units; scFv, single-chain Fv antibody fragments; TPBS, 0.05% (vol/vol) Tween 20 in PBS; TMPBS, 0.05% (vol/vol) Tween 20 in MPBS; TU, transducing units; V_H, immunoglobulin heavy-chain variable region; V_κ, immunoglobulin kappa light-chain variable region; V_L, immunoglobulin light-chain variable region.

Oligonucleotide design. Family-specific murine V_H and V_κ primers were designed as previously described for human V-gene primers (35, 36) to amplify full-length rearranged V genes. Briefly, murine V_H and V_κ DNA sequences were collected from the Kabat et al. (30) and GenBank databases, aligned, and classified by family, and family-specific primers were designed to anneal to the first 23 nucleotides comprising framework 1. Similarly, J_H and J_κ gene-segment-specific primers were designed to anneal to the final 24 nucleotides comprising each of the 4 J_H and 5 J_κ gene segments (30).

Vector construction. To construct the vector pSYN3, a 1.5-kb stuffer fragment was amplified from pCANTAB5E (Pharmacia Biotech, Milwaukee, Wis.) by using PCR and the primers LMB3 (35) and E-tagback (5'-ACCACCGAATTCTATTAATGGTGATGATGGTGGATGACCAGCCGGTTCCAGCGG-3'). The DNA fragment was digested with *Sfi*I and *Not*I, gel purified, and ligated into pCANTAB5E digested with *Sfi*I and *Not*I. Ligated DNA was used to transform *Escherichia coli* TG1 (19), and clones containing the correct insert were identified by DNA sequencing. The resulting vector permits subcloning of phage-displayed scFv as *Sfi*I-*Not*I or *Nco*I-*Not*I fragments for secretion into the periplasm of *E. coli* as native scFv with a C-terminal E epitope tag followed by a hexahistidine tag.

Immunizations. For construction of library 1, BALB/c mice (16 to 22 g) were immunized at 0, 2, and 4 weeks with pure BoNT/A H_C (Ophidian Pharmaceuticals, Madison, Wis.). Each animal was given subcutaneously 1 μg of material adsorbed onto alum (Pierce Chemical Co., Rockford, Ill.) in a volume of 0.5 ml. Mice were challenged 2 weeks after the second immunization with 100,000 50% lethal doses of pure BoNT/A and were sacrificed 1 week later. For construction of library 2, CD-1 mice (16 to 22 g) were immunized at 0, 2, and 4 weeks with pure BoNT/A H_C and were sacrificed 2 weeks after the third immunization. For both libraries, the spleens were removed immediately after sacrifice and total RNA was extracted by the method of Cathala et al. (7).

Library construction. First-strand cDNA was synthesized from approximately 10 μg of total RNA exactly as previously described (35), except that immunoglobulin mRNA was specifically primed with 10 pmol each of MlgG1 For, MlgG3 For, and MC_κ For (Table 1). For construction of library 1, rearranged V_H and V_κ genes were amplified from first-strand cDNA by using commercially available V_H and V_κ back primers and J_H and J_κ forward primers (Recombinant Phage Antibody System; Pharmacia Biotech). For library 2, equimolar mixtures of family-specific V_H and V_κ back primers were used in conjunction with equimolar mixtures of J_H or J_κ gene-segment-specific forward primers in an attempt to increase library diversity (see "Oligonucleotide design" above). Rearranged V_H and V_κ genes were amplified separately in 50-μl reaction mixtures containing 5 μl of the first-strand cDNA reaction mixture, 20 pmol of an equimolar mixture of the appropriate back primers, 20 pmol of an equimolar mixture of the appropriate forward primers, 250 μM (each) deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10 μg of bovine serum albumin/ml, and 1 μl (5 U) of *Thermus aquaticus* (*Taq*) DNA polymerase (Promega) in the buffer supplied by the manufacturer. The reaction mixture was overlaid with paraffin oil (Sigma) and cycled 30 times (at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min). Reaction products were gel purified, isolated from the gel by using DEAE membranes, eluted from the membranes with high-salt buffer, ethanol precipitated, and resuspended in 20 μl of water (43).

scFv gene repertoires were assembled from purified V_H and V_κ gene repertoires and linker DNA by using splicing by overlap extension. Linker DNA encoded the peptide sequence $(G_4S)_3$ (27) and was complementary to the 3' ends of the rearranged V_H genes and the 5' ends of the rearranged V_κ genes. The V_H and V_κ DNAs (1.5 μg of each) were combined with 500 ng of linker DNA (Recombinant Phage Antibody System; Pharmacia Biotech) in a 25-μl PCR mixture containing 250 μM (each) deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10 μg of bovine serum albumin/ml, and 1 μl (5 U) of *Taq* DNA polymerase (Promega) in the buffer supplied by the manufacturer, and the mixture was cycled 10 times (at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min) to join the fragments. Flanking oligonucleotide primers (RS, provided in the Recombinant Phage Antibody System kit, for library 1 and an equimolar mixture of V_HSfi and J_κNot primers (Table 1) for library 2) were added, and the reaction mixture was cycled for 33 cycles (at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min) to append restriction sites. scFv gene repertoires were gel purified as described above, digested with *Sfi*I and *Not*I, and purified by electroelution, and 1 μg of each repertoire was ligated into either 1 μg of pCANTAB5E vector (Pharmacia Biotech) (library 1) or 1 μg of pHEN-1 (25) (library 2) digested with *Sfi*I and *Not*I. The ligation mix was purified by extraction with phenol-chloroform, ethanol precipitated, and resuspended in 20 μl of water, and 2.5-μl samples were electroporated (16) into 50 μl of *E. coli* TG1 (19). Cells were grown in 1 ml of SOC (43) for 30 min and then plated on TYE (39) medium containing 100 μg of AMP/ml and 1% (wt/vol) GLU (TYE-AMP-GLU). Colonies were scraped off the plates into 5 ml of 2× TY broth (39) containing 100 μg of AMP/ml, 1% GLU (2× TY-AMP-GLU), and 15% (vol/vol) glycerol for storage at −70°C. The cloning efficiency and diversity of the libraries were determined by PCR screening (20) exactly as described in reference 36.

Preparation of phage. To rescue phagemid particles from the libraries, 10 ml of 2× TY-AMP-GLU was inoculated with an appropriate volume of bacteria (approximately 50 to 100 μl) from the library stocks to give an A_{600} of 0.3 to 0.5 and bacteria were grown for 30 min with shaking at 37°C. About 10^{12} PFU of VCS-M13 (Stratagene) particles were added, and the mixture was incubated at

TABLE 1. Oligonucleotide primers used for PCR of mouse immunoglobulin genes

Procedure	Primer	Sequence[a]
1st-strand cDNA synthesis	Heavy-chain constant region	
	MIgG1/2 For	5' CTG GAC AGG GAT CCA GAG TTC CA 3'
	MIgG3 For	5' CTG GAC AGG GCT CCA TAG TTC CA 3'
	κ constant region, $MC_κ$ For	5' CTC ATT CCT GTT GAA GCT CTT GAC 3'
Primary PCR	V_H back	
	V_H1 Back	5' GAG GTG CAG CTT CAG GAG TCA GG 3'
	V_H2 Back	5' GAT GTG CAG CTT CAG GAG TCR GG 3'
	V_H3 Back	5' CAG GTG CAG CTG AAG SAG TCA GG 3'
	V_H4/6 Back	5' GAG GTY CAG CTG CAR CAR TCT GG 3'
	V_H5/9 Back	5' CAG GTY CAR CTG CAG CAG YCT GG 3'
	V_H7 Back	5' GAR GTG AAG CTG GTG GAR TCT GG 3'
	V_H8 Back	5' GAG GTT CAG CTT CAG CAG TCT GG 3'
	V_H10 Back	5' GAA GTG CAG CTG KTG GAG WCT GG 3'
	V_H11 Back	5' CAG ATC CAG TTG CTG CAG TCT GG 3'
	$V_κ$ back	
	$V_κ$1 Back	5' GAC ATT GTG ATG WCA CAG TCT CC 3'
	$V_κ$2 Back	5' GAT GTT KTG ATG ACC CAA ACT CC 3'
	$V_κ$3 Back	5' GAT ATT GTG ATR ACB CAG GCW GC 3'
	$V_κ$4 Back	5' GAC ATT GTG CTG ACM CAR TCT CC 3'
	$V_κ$5 Back	5' SAA AWT GTK CTC ACC CAG TCT CC 3'
	$V_κ$6 Back	5' GAY ATY VWG ATG ACM CAG WCT CC 3'
	$V_κ$7 Back	5' CAA ATT GTT CTC ACC CAG TCT CC 3'
	$V_κ$8 Back	5' TCA TTA TTG CAG GTG CTT GTG GG 3'
	J_H forward	
	J_H1 For	5' TGA GGA GAC GGT GAC CGT GGT CCC 3'
	J_H2 For	5' TGA GGA GAC TGT GAG AGT GGT GCC 3'
	J_H3 For	5' TGC AGA GAC AGT GAC CAG AGT CCC 3'
	J_H4 For	5' TGA GGA GAC GGT GAC TGA GGT TCC 3'
	$J_κ$ forward	
	$J_κ$1 For	5' TTT GAT TTC CAG CTT GGT GCC TCC 3'
	$J_κ$2 For	5' TTT TAT TTC CAG CTT GGT CCC CCC 3'
	$J_κ$3 For	5' TTT TAT TTC CAG TCT GGT CCC ATC 3'
	$J_κ$4 For	5' TTT TAT TTC CAA CTT TGT CCC CGA 3'
	$J_κ$5 For	5' TTT CAG CTC CAG CTT GGT CCC AGC 3'
Reamplification	Restriction site containing	
	V_H *Sfi* back	
	V_H1 Sfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTT CAG GAG TCA GG 3'
	V_H2 Sfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAT GTG CAG CTT CAG GAG TCR GG 3'
	V_H3 Sfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG AAG SAG TCA GG 3'
	V_H4/6 Sfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTY CAG CTG CAR CAR TCT GG 3'
	V_H5/9 Sfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTY CAR CTG CAG CAG YCT GG 3'
	V_H7 Sfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAR GTG AAG CTG GTG GAR TCT GG 3'
	V_H8 Sfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTT CAG CTT CAG CAG TCT GG 3'
	V_H10 Sfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAA GTG CAG CTG KTG GAG WCT GG 3'
	V_H11 Sfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG ATC CAG TTG CTG CAG TCT GG 3'
	$J_κ$ *Not* forward	
	$J_κ$1 Not	5' GAG TCA TTC TCG ACT TGC GGC CGC TTT GAT TTC CAG CTT GGT GCC TCC 3'
	$J_κ$2 Not	5' GAG TCA TTC TCG ACT TGC GGC CGC TTT TAT TTC CAG CTT GGT CCC CCC 3'
	$J_κ$3 Not	5' GAG TCA TTC TCG ACT TGC GGC CGC TTT TAT TTC CAG TCT GGT CCC ATC 3'
	$J_κ$4 Not	5' GAG TCA TTC TCG ACT TGC GGC CGC TTT TAT TTC CAA CTT TGT CCC CGA 3'
	$J_κ$5 Not	5' GAG TCA TTC TCG ACT TGC GGC CGC TTT CAG CTC CAG CTT GGT CCC AGC 3'

[a] R, A/G; Y, C/T, S, G/C; K, G/T; W, A/T; M, A/C; V, C/G/A; B, G/C/T; H, C/A/T.

37°C for 30 min without shaking, followed by incubation at 37°C for 30 min with shaking. Cells were spun down, resuspended in 50 ml of 2× TY broth containing 100 μg of AMP/ml and 50 μg of KAN/ml (2× TY-AMP-KAN), and grown overnight with shaking at 25°C. Phage particles were purified and concentrated by two polyethylene glycol precipitations (43), resuspended in 5 ml of PBS, and filtered through a 0.45-μm-pore-size filter. The phage preparation consistently resulted in a titer of approximately 10^{13} TU of AMP-resistant clones/ml.

Selection of phage antibody libraries. Both libraries were selected by using 75- by 12-mm immunotubes (Maxisorp; Nunc) coated with 1 ml of BoNT/A (50 μg/ml; kindly provided by Ray Stevens) or BoNT/A H_C (10 μg/ml) in PBS

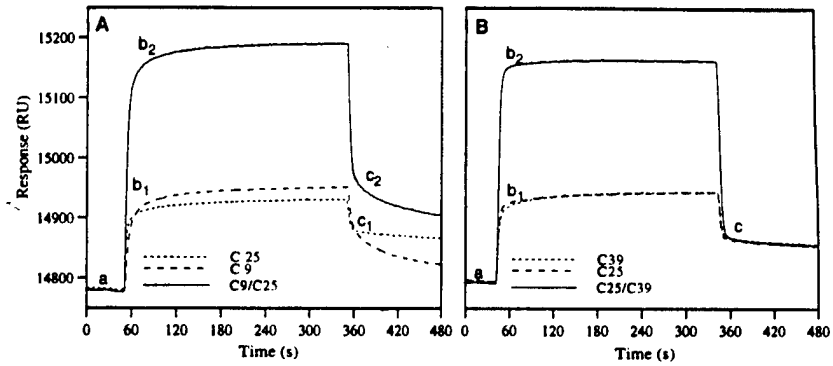

FIG. 2. Sensorgrams illustrating technique used to epitope map scFv binding to BoNT/A H_C. Epitope mapping was performed by using surface plasmon resonance in a BIAcore, with scFv studied in pairs. Each scFv was injected into the BIAcore and allowed to bind to BoNT/A H_C coupled to the sensor chip surface until saturation was achieved. The amount (in RU) bound for each scFv alone was compared to the amount bound when the two scFv were mixed and injected together. Point a, baseline, followed by the beginning of injection; points b_1 and b_2, initial association phase; points c_1 and c_2, beginning of dissociation. The differences in RU between points a and c equal the amount of scFv bound to BoNT/A H_C. (A) Two scFv recognizing different epitopes are studied (C25 and C9). The amount bound for the two scFv injected together (C9/C25; point c_2) is the sum of the two scFv injected alone (c_1). (B) Two scFv recognizing the same epitope are studied (C39 and C25). The amount bound for the two scFv injected together (C25/C39; point c) is the same as that for the two scFv injected alone (c). The large differences in RU between points b_1 and c_1, b_2 and c_2, and b_1 and c are due to differences in refractive index between scFv and running buffer.

overnight at 4°C. Tubes were blocked for 1 h at 37°C with 2% MPBS, and selection, washing, and elution were performed exactly as described in reference 35 by using phage at a concentration of 5.0×10^{12} TU/ml. One-third of the eluted phage was used to infect 10 ml of log-phase *E. coli* TG1, which was plated on TYE-AMP-GLU plates as described above. The rescue-selection-plating cycle was repeated three times, after which clones were analyzed for binding by ELISA. Libraries were also selected on soluble BoNT/A H_C. For library 1, 1.0 mg of BoNT/A H_C (700 μg/ml) was biotinylated (Recombinant Phage Selection Module; Pharmacia) and purified as recommended by the manufacturer. For each round of selection, 1 ml of phage (approximately 10^{13} TU) were mixed with 1 ml of PBS containing 4% skim milk powder, 0.05% Tween 20, and 10 μg of biotinylated BoNT/A H_C/ml. After 1 h at room temperature, antigen-bound phage were captured on blocked streptavidin-coated M280 magnetic beads (Dynabeads; Dynal) exactly as described in reference 45. Dynabeads were washed a total of 10 times (three times in TPBS, twice in TMPBS, twice in PBS, once in MPBS, and two more times in PBS). Bound phage were eluted from the Dynabeads by incubation with 100 μl of 100 mM triethylamine for 5 min and were neutralized with 1 M Tris-HCl, pH 7.5, and one-third of the eluate was used to infect log-phase *E. coli* TG1. For library 2, affinity-driven selections (22, 45) were performed by decreasing the concentration of soluble BoNT/A H_C used for selection (10 μg/ml for round 1, 1 μg/ml for round 2, and 10 ng/ml for round 3). Soluble BoNT/A H_C was captured on 200 μl of Ni^{2+}-NTA (Qiagen) via a C-terminal hexahistidine tag. After capture, the Ni^{2+}-NTA resin was washed a total of 10 times (5 times in TPBS and 5 times in PBS), bound phage were eluted as described above, and the eluate was used to infect log-phase *E. coli* TG1.

Initial characterization of binders. Initial analysis for binding to BoNT/A, BoNT/A H_C, and BoNT/A H_N (kindly provided by Ray Stevens) (8) was performed by ELISA using bacterial supernatant containing expressed scFv. Expression of scFv (13) was performed in 96-well microtiter plates exactly as described in reference 35. For ELISA, microtiter plates (Falcon 3912) were coated overnight at 4°C with either BoNT/A, BoNT/A H_C, or BoNT/A H_N (10 μg/ml) in PBS and then were blocked with 2% MPBS for 1 h at room temperature. Bacterial supernatants containing expressed scFv were added to wells and incubated at room temperature for 1.5 h. Plates were washed six times (3 times with TPBS and 3 times with PBS), and binding of scFv was detected via their C-terminal peptide tags (E epitope tag for library 1 in pCANTAB5E and myc epitope tag [42] for library 2 in pHEN-1) by using either anti-myc tag antibody (9E10; Santa Cruz Biotechnology) or anti-E antibody (Pharmacia Biotech) and peroxidase-conjugated anti-mouse Fc antibody (Sigma), as described in references 35 and 44. The number of unique binding scFv was determined by *Bst*N1 fingerprinting and DNA sequencing.

Subcloning, expression, and purification of scFv. To facilitate purification, scFv genes were subcloned into the expression vector pUC119mycHis (46) or pSYN3, resulting in the addition of a hexahistidine tag at the C-terminal end of the scFv. Two-hundred-milliliter cultures of *E. coli* TG1 harboring one of the appropriate phagemids were grown, expression of scFv was induced with IPTG (13), and the cultures were grown at 25°C overnight. scFv was harvested from the periplasm (6), dialyzed overnight at 4°C against IMAC loading buffer (50 mM sodium phosphate [pH 7.5], 500 mM NaCl, 20 mM imidazole), and then filtered through a 0.2-μm-pore-size filter. scFv was purified by IMAC (24) exactly as described in reference 46. To separate monomeric scFv from dimeric and aggregated scFv, samples were concentrated to a volume of <1 ml in a centrifugal concentrator (Centricon 10; Amicon) and fractionated on a Superdex 75 column (Pharmacia) by using HBS. The purity of the final preparation was evaluated by assaying an aliquot by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein bands were detected by Coomassie blue staining. The concentration was determined spectrophotometrically, on the assumption that an A_{280} of 1.0 corresponds to an scFv concentration of 0.7 mg/ml.

Measurement of affinity and binding kinetics. The K_ds of purified scFv were determined by using surface plasmon resonance in a BIAcore (Pharmacia Biosensor AB). In a BIAcore flow cell, approximately 600 RU of BoNT/A H_C (15 μg/ml in 10 mM sodium acetate [pH 4.5]) was coupled to a CM5 sensor chip by using *N*-hydroxysuccinimide–*N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide chemistry (29). This amount of coupled BoNT/A H_C resulted in a maximum RU of 100 to 175 of scFv bound. For regeneration of the surface after binding of scFv, 5 μl of 4 M $MgCl_2$ was injected, resulting in a return to baseline. The surface was reused 20 to 30 times under these regeneration conditions. Association was measured under a continuous flow of 5 μl/min with a concentration range from 50 to 1,000 nM. k_{on} was determined from a plot of ln $(dR/dt)/t$ versus concentration, where R is response and t is time (31). k_{off} was determined from the dissociation part of the sensorgram at the highest concentration of scFv analyzed (31) by using a flow rate of 30 μl/min. K_d was calculated as k_{off}/k_{on}.

Epitope mapping. Epitope mapping was performed by using surface plasmon resonance in a BIAcore. In a BIAcore flow cell, approximately 1,200 RU of BoNT/A H_C was coupled to a CM5 sensor chip as described above. With a flow rate of 5 μl/min, a titration of 100 nM to 1 μM scFv was injected over the flow cell surface for 5 min to determine an scFv concentration which resulted in near saturation of the surface. Epitope mapping was performed with pairs of scFv at concentrations resulting in near saturation and at least 100 RU of scFv bound. The amount of scFv bound was determined for each member of a pair, and then the two scFv were mixed together to give a final concentration equal to the concentration used for measurements of the individual scFv. scFv recognizing different epitopes showed an additive increase in the RU bound when injected together (Fig. 2A), while scFv recognizing identical epitopes showed only a minimal increase in RU (Fig. 2B).

In vitro neutralization studies. In vitro neutralization studies were performed by using a mouse hemidiaphragm preparation, as described by Deshpande et al. (14). Briefly, left and right phrenic nerve hemidiaphragm preparations were excised from male CD/1 mice (25 to 33 g) and suspended in physiological solution (135 mM NaCl, 5 mM KCl, 15 mM $NaHCO_3$, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 11 mM GLU). The incubation bath was bubbled with 95% O_2–5% CO_2 and maintained at a constant temperature of 36°C. Phrenic nerves were stimulated supramaximally at 0.05 Hz with square waves of 0.2 ms duration. Isometric twitch tension was measured with a force displacement transducer (Model FT03; Grass) connected to a chart recorder. Purified scFv were incubated with purified BoNT/A for 30 min at room temperature and then added to the tissue bath, resulting in a final scFv concentration of 2.0×10^{-8} M and a final BoNT/A concentration of 2.0×10^{-11} M. For each scFv studied, time to 50% twitch tension reduction was determined three times for BoNT/A alone and three times for scFv plus BoNT/A. The combination of S25 and C25 was studied at a final concentration of 2.0×10^{-8} M each. Differences between times to 50% twitch reduction were determined by a two-tailed t test, with a P value of <0.05 considered significant.

TABLE 2. Frequency of binding of clones from phage antibody libraries

Antigen used for selection	Frequency of ELISA-positive clones[a] in selection round:		
	1	2	3
Library 1[b]			
BoNT/A: immunotube[c]	20/184	124/184	ND
BoNT/A H_C: immunotube	7/92	86/92	88/92
BoNT/A H_C: biotinylated[d]	7/90	90/90	90/90
	14/48	48/48	ND
Library 2[e]			
BoNT/A: immunotube	ND	81/92	ND
BoNT/A H_C: immunotube	ND	ND	76/92
BoNT/A H_C: Ni^{2+}-NTA[f]	ND	ND	67/92

[a] Expressed as number of positive clones/total number of clones. For selections on BoNT/A and BoNT/A H_C, ELISA was done on immobilized BoNT/A and BoNT/A H_C, respectively. ND, data not determined from selection performed.

[b] Derived from a mouse immunized twice with BoNT/A H_C and once with BoNT/A.

[c] Immunotube selections were performed with the antigen absorbed onto immunotubes.

[d] Biotinylated selections were performed in solution with capture on streptavidin magnetic beads.

[e] Derived from a mouse immunized three times with BoNT/A H_C.

[f] Ni^{2+}-NTA selections were performed in solution with capture on Ni^{2+}-NTA agarose.

RESULTS

Phage antibody library construction and characterization. Two phage antibody libraries were constructed from the V_H and V_κ genes of immunized mice (Fig. 1). For library 1, a mouse was immunized twice with BoNT/A H_C and challenged 2 weeks after the second immunization with 100,000 50% lethal doses of BoNT/A. The mouse survived the BoNT/A challenge and was sacrificed 1 week later. The spleen was removed immediately after sacrifice, and total RNA was prepared. For library construction, IgG heavy-chain and kappa light-chain mRNA were specifically primed and first-strand cDNA was synthesized. V_H and V_κ gene repertoires were amplified by PCR, and V_H, J_H, V_κ, and J_κ primers were provided in the recombinant phage antibody system. The V_H and V_κ gene repertoires were randomly spliced together to create an scFv gene repertoire by using synthetic DNA encoding the 15-amino-acid peptide linker $(G_4S)_3$. Each scFv gene repertoire was separately cloned into the phage display vector pCANTAB5E (Pharmacia). After transformation, a library of 2.1×10^6 members was obtained. Ninety percent of the clones had an insert of the appropriate size for an scFv gene, as determined by PCR screening, and the cloned scFv genes were diverse, as determined by PCR fingerprinting (data not shown). DNA sequencing of 10 unselected clones from library 1 revealed that all V_H genes were derived from the murine V_H2 family and all V_κ genes were derived from the murine $V_\kappa4$ and $V_\kappa6$ families (30). Based on this observed V-gene bias, family-specific V_H and V_κ primers were designed along with J_H and J_κ gene-segment-specific primers (Table 1). These primers were then used to construct a second phage antibody library. For library 2, a mouse was immunized three times with BoNT/A H_C and sacrificed 2 weeks after the third immunization. The mouse was not challenged with BoNT/A prior to spleen harvest, as this led to the production of non-H_C-binding antibodies (see "Selection and initial characterization of phage antibodies" below). The spleen was harvested, and a phage antibody library was constructed as described above, except that V_H-, J_H-, V_κ-, and J_κ-specific primers were used. After transformation, a library of 1.0×10^6 members was obtained. Ninety-five percent of the clones had an insert of the appropriate size for an scFv gene, as determined by PCR screening, and the cloned scFv genes were diverse, as determined by PCR fingerprinting (data not shown). DNA sequencing of 10 unselected clones from library 2 revealed greater diversity than was observed in library 1; V_H genes were derived from the V_H1, V_H2, and V_H3 families, and V_κ genes were derived from the $V_\kappa2$, $V_\kappa3$, $V_\kappa4$, and $V_\kappa6$ families (30).

Selection and initial characterization of phage antibodies. To isolate BoNT/A binding phage antibodies, phage were rescued from the library and selected on either purified BoNT/A or BoNT/A H_C. Selections were performed on the holotoxin in addition to H_C, since it was unclear to what extent the recombinant toxin H_C would mimic the conformation of the H_C in the holotoxin. Selection for BoNT/A and BoNT/A H_C binders was performed on antigen adsorbed to polystyrene. In addition, H_C binding phage were selected in solution on biotinylated H_C, with capture on streptavidin magnetic beads (for library 1) or on hexahistidine tagged H_C, with capture on Ni^{2+}-NTA agarose (for library 2). Selections in solution were utilized based on our previous observation that selection on protein adsorbed to polystyrene could yield phage antibodies which did not recognize native protein (46). Selection in solution was not performed on the holotoxin due to our inability to successfully biotinylate the toxin without destroying immunoreactivity.

After two to three rounds of selection, at least 67% of scFv analyzed bound the antigen used for selection (Table 2). The number of unique scFv was determined by DNA fingerprinting followed by DNA sequencing, and the specificity of each scFv was determined by ELISA on pure BoNT/A and recombinant BoNT/A H_C and H_N. scFv binding BoNT/A but not binding H_C or H_N were presumed to bind the light chain (catalytic domain). A total of 33 unique scFv were isolated from mice immunized with H_C and challenged with BoNT/A (Table 3, library 1). When library 1 was selected on holotoxin, 25 unique scFv were identified. Only 2 of these scFv, however, bound H_C, with the majority (21) binding the light chain and 2 binding H_N. The two H_C binding scFv did not express as well as other scFv recognizing similar epitopes, and they were therefore not characterized with respect to affinity or neutralization capacity (see below). Selection of library 1 on H_C yielded an additional eight unique scFv (Tables 3 and 4). Overall, however, only 50% of scFv selected on H_C also bound holotoxin. This result suggests that a significant portion of the H_C surface may be inaccessible in the holotoxin. Alternatively, scFv could be binding H_C conformations that do not exist in the holotoxin. From mice immunized with H_C only (library 2), all scFv selected on holotoxin also bound H_C. As with library 1, however, only 50% of scFv selected on H_C bound holotoxin. In all, 18 unique H_C binding scFv were isolated from library 2, resulting in a total of 28 unique H_C binding scFv (Tables 3 and 4). scFv of identical

TABLE 3. Specificity of BoNT binding scFv selected from phage antibody libraries

scFv specificity	No. of unique scFv in:	
	Library 1	Library 2
BoNT/A H_C	10	18
BoNT/A H_N	2	0
BoNT/A light chain	21	0
Total	33	18

TABLE 4. Deduced protein sequences of V_H and V_L of BoNT/A H_C binding scFv, classified by epitope recognized

Region	Epitope	Clone	Lib[a]	Sequence[b]						
				Framework 1	CDR 1	Framework 2	CDR 2	Framework 3	CDR 3	Framework 4
V_H	1	C15	1	QVKLQQSGAELVRPGASVKLSCKTSGYSFT	SYWMN	WVKQGPGQGLEWIG	MIHPSNSEIRFNQKFED	MATLTVDKSSSTAYMQLSSPTSEDSAVYYCAR	GIYYDYDGGNYYAMDY	WGQGTTVTASS
		C9	1	----------	-----	----------	----------N	----------	----V-----------	--------V--
		1D5	2	E---VE----------N----A------	-----	----R---------	--------T-L----K-	K----------	-------E-Y--TL--	------L-V--
		C1 .	1	----------A------	-----	----R---------	-------DT--------	K------R-----IH----------	-L-GYGF WYFDV	--------V--
		S25	1	----------A----L-	-----	----R---------	-----D-DT--------	K------T----------	-L-NGF WYF-V	--------V--
		1B6	2	--Q----------V---I---A---T-I	D-A-H	----S-AKS-----	V-SSYYGDTDY--I-KG	K---------N----E-ARL--D---I-----	RGKG ----	--------V--
		1C9	2	--Q-K----------V---I---G---T-I	D-AVH	----SHAKS-----	V-STYYGDADY-PK-KG	K-----N---N----E-PRL------I-----	RGKG ----	-----S--V--
		1E8	2	E-Q--E--PG--K-SQ-LS-T-TVT---I-	D-AW-	-IR-F--KK---M-	Y-S YSGSTGYNPSLKS	RISI-R-T-KNQFFL--N-V-T--TGT-----	-YD ----	-----S--V--
		1G7	2	E-Q--E--PG -K-SQ-LS-T-TVT---I-	D-AWY	-IR-F--KK---M-	Y-S YSGSTGYNPSLKS	RISI-R-T-KNQFFL--N-V-T--TGT-----	-YD ----	-----S--V--
	2	1A1	2	EVKLVESGGGLVQPGGSRKLSCATSGFTFS	DYYMS	WIRQSPDKRLEWVA	TISDGGTYTYYPDSVKG	RFTISRDNAKNTLYLQMSSLKSEDTAMYYCVR	HGYGNYPSH WYFDV	WGAGTTVTVSS
		1F1	2	----------L-----A------	N-G--	-V--T---------	M--S--S-N--S-----	-V--------S---------Q-------L-T-	--------Y -----	----------
		C39	1	Q-Q-Q-----S-K----L-----A------	-----	-V--T-E-------	------S----------	----------N----------I-----	YR-DEGL -Y	--Q--------
		C25	1	Q-Q-Q-------K----L-----A------	----Y	-V--T-E-------	------S----------	----------N----------S-	YR-DDAM -Y	--Q--------
		2G5	2	----------K----L-----A------	S-A--	-V--T-E-------	----------T-N---	----------HN------H----------A-	NLPYDHV -Y	--Q--S-----
		3C3	2	----K----------K----L-----A------	S-A--	-V--T-E-------	----------T-N---	----------HN-------H----------A-	NLPYDHV -Y	--Q--S-----
		3F4	2	EG----------K----L-----A------	S-A--	-V--T-EH------	-------F---T-N---	----------HN------H----------A-	NLPYDHV -Y	--Q--S-----
		3H4	2	----------K---PL-----A------	S-A--	-V--T-EH------	-------F---T-N---	----------HN------H----------A-	NLPYDHV -Y	--Q--S-----
	3	1B3	2	EVQLQESGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	DWSEGYYYYG MDV	WGQGTTVIVSS
		1C6	2	QI--LQ----------	-----	----------	----------	----------	---------- ---	----------
		2B6	2	VKLVESGP-L-KPSQSLSLTCTVTGYSIT-	D-AWN	-I--F--NK---MG	Y-N-----N-NP -L-N	-ISIT--T---QFF-KL--VTS----T-----	AGDGY-VD WYFDV	--T--------
		1G5	2	Q----Q--AEL----A-VKM--K---Y--T	--WTT	--K-R--Q----IG	D-YPGSGSTNYNEKF-S	KA-LTV-T-SS-A-M-LS--TS--S-------	ELGD A--Y	-----S-----
		1H6	2	-----Q--AEL-K--A-VKM--K---Y--T	--WTT	--K-R--Q----IG	D-YP-SGSTNYNEKF-S	KA-LTV-T-SS-A-M-LS--TS--S-------	ELGD A--Y	-----S-----
	4	1F3	2	EVQLQQSGAELVKPGASVKLSCKASGYTFT	SFWMH	WVKQRPGRGLEWIG	RLDPNSGETKYNEKFKS	KATLTVDKPSSTAYMELSSLTSEDSAVYYCAR	EAYGYWN FDV	WGTGTTVTVSS
		2E8	2	----------	-----	----------	----------K----	----------	------- ---	----------
V_L	1	C15	1	DIELTQSPAIMSASPGEKVIMTC	SASS SVSHMY	WYQQKPGSSPRLLIY	DTSNLAS	GVPIRFSGSGSGTSYSLTISRMEAEDSATYYC	QQWSSYPFT	FGSGTKLELKR
		C9	1	--D---------S-------I--	---- ---Y-H	-F-----T--KPW--	S------	---A----------SV----A-----	--Y-G--L-	--A-----I--
		1D5	2	----------A--------I--	----S I-S-NLH	-----SETSPKPW--	G------	---V----------S-----A-----	---G---L-	--G-----I--
		C1	1	----------	---- ---Y--	----------	-------	---V----------A-----	-------L-	--A--------
		S25	1	---------L-A--------I--	-V--S I-S-NLH	-----S-T--KPW--	G------	---V----------S-----A-----	-------L-	--A-----I--
		1B6	2	---------SLAV-L-QRA-IS-	RA-ESVDSYGN-F-H	-------QP-K----	RA---E-	-I-A-------R-DFT--INPV--D-V-----	--SNED-P-	--A--------
		1C9	2	---------SLAV-L-QRA-IS-	RA-ESVDSYGN-F-H	-------QP-K----	RA---E-	-I-A-------R-DFT---NPV--D-V-----	--SNED-Y-	--G-----I--
		1E8	2	----------	---- ---Y-H	-----S-T--KRW--	---K---	---A----------S-----A-----	-----N-L-	--A--------
		1G7	2	----------	---- ---Y-H	-----S-T--KRW--	---K---	---A----------S-----A-----	-----N-L-	--A--------
	2	1A1	2	DIELTQSPASLAVSLGQRATISC	RASESVDSYGNSFMH	WYQQKPGQPPKLLIY	LASNLES	GVPARFSGSGSRTDFTLTIDPVEADDAATYYC	QQNNEDPYT	FGGGTKLEIKR
		1F1	2	--------T----------	----------	----------	-------	----------	---------	----------
		C39	1	----------R------	----------H----	----------	-------	----------	---------	----------
		C25	1	----------	----------H---Q	----------	R-----P	-I---------G-------N------V-----	--S----F-	--S--------
		2G5	2	---------IMSA-P-EKVTTT-	S--S SV-Y--	-F-----TS-K-W--	ST---A-	----------G-SYS---SRM--E-------	--RSSY---	----DQAGN-S
		3C3	2	---------IMSA-P-EKVTTT-	----------H---Q	-F-----TS-K-W--	ST---A-	----------G-SYS---SRM--E-------	--RSSY---	----DQAGN--
		3F4	2	-T--------IMSA-P-EKVTMT-	S--S SV-Y-Y	-------SS-R----	DT---A-	---V-------G-SYS---SRM--E-------	--WSSY-P-	----------
		3H4	2	---------IMSA-P-EKVTMT-	---S- VSS-YL-	-------SS-R----	DT---A-	---V-------G-SYS---SRM--E-------	--WSSY-P-	----------
	3	1B3	2	DSELTQSPTTMAASPGEKITTTC	SASSS ISSNYLH	WYQQRPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPRT	FGGGTKLEIKR
		1C6	2	-I------ASL-V-L-RRA--S-	R--E-VEYYGTSLMQ	----K--QP------	AA--VE-	----------	--SRKV-W-	----------
		2B6	2	YI------ASL-V-L-QRA--S-	R--E-VDSYGNSFM-	----K--QP------	LA---E-	----------R-DFT---DPV--D-A-----	--NNED-Y-	----------S
		1G5	2	-I------ASL-V-L-QRA--S-	R--E-VEYYGTSLMQ	----K--QP------	AA--VE-	-A----------DF--N-HPV-ED-I-M-F-	--SRKV-Y-	----------
		1H6	2	-I------AI-S------V----	-V--- ---SN--	----KS-T----W--	G------	---V----------SS-----A-----	--W--Y-L-	--A---V-LR-
	4	1F3	2	DIELTQSPASMSASPGEKVTMTC	RATSS VSSSYLH	WYQQKSGASPKLWIY	SASNLAS	GVPSRFSGSGSGTSYSLTISSVEAEDAATYYC	QQYIGYPYT	FGGGTKLEIKR
		2E8	2	--------TT-A------I-I--	S-S-- IG-N---	-----P-F----L--	RT-----	---A----------GAM----V-----	--GSSI---	----------

[a] Lib, library.

[b] Full-length sequences were not determined for clones C12, C13, C2, and S44 (all bind epitope 1). Accession can be made through GenBank with nos. AF003702 to AF003725.

or related sequences were isolated on both H_C immobilized on polystyrene and H_C in solution. Thus, in the case of H_C, the method of selection was not important.

Epitope mapping. All 28 unique H_C binding scFv were epitope mapped by using surface plasmon resonance in a BIAcore. Epitope mapping was performed with pairs of scFv at concentrations resulting in near saturation of the chip surface and at least 100 RU of scFv bound. The amount of scFv bound was determined for each member of a pair, and then the two scFv were mixed together to give a final concentration equal to the concentration used for measurements of the individual scFv. scFv recognizing different epitopes showed an additive increase in the RU bound when injected together (Fig. 2A), while scFv recognizing identical epitopes showed only a minimal increase in RU (Fig. 2B). By this technique, mapping of the 28 scFv yielded 4 nonoverlapping epitopes recognized on H_C (Table 4). scFv recognizing only epitopes 1 and 2 were obtained from library 1, whereas scFv recognizing all 4 epitopes were obtained from library 2. Many of the scFv recognizing the same epitope (C1 and S25; C9 and C15; 1E8 and 1G7; 1B6 and 1C9; C25 and C39; 2G5, 3C3, 3F4, and 3H4; 1A1 and 1F1; 1B3 and 1C6; 1G5 and 1H6; 1F3 and 2E8) had V_H domains derived from the same V-D-J rearrangement, as evidenced by the high level of homology of the V_H CDR3 and V_H-gene segment (Table 4). These scFv differ only by substitutions introduced by somatic hypermutation or PCR error. For epitopes 1 and 2, most or all of the scFv recognizing the same epitope are derived from the same or very similar V_H-gene segments but differ significantly with respect to V_H CDR3 length and sequence (5 of 9 scFv for epitope 1; 8 of 8 scFv for epitope 2) (Table 4). These include scFv derived from different mice. Given the great degree of diversity in V_H CDR2 sequences in the primary repertoire (49), specific V_H-gene segments may have evolved for their ability to form binding sites capable of recognizing specific pathogenic antigenic shapes. In contrast, greater structural variation appears to occur in the rearranged V_κ genes. For example, three different germ line genes and CDR1 main-chain conformations (9) are observed for epitope 2, where all the V_H genes are derived from the same germ line gene. Such "promiscuity" in chain pairings has been reported previously (10).

Affinity, binding kinetics, and in vitro toxin neutralization. Affinity, binding kinetics, and in vitro toxin neutralization were determined for one representative scFv binding to each epitope. For each epitope, the scFv chosen for further study had the best combination of high expression level and slow k_{off}, as determined during epitope mapping studies. K_d for the four scFv studied ranged between 7.3×10^{-8} and 1.1×10^{-9} M (Table 5), values comparable to those reported for monoclonal IgG produced from hybridomas (18). C25 has the highest affinity ($K_d = 1.1 \times 10^{-9}$ M) reported for an anti-botulinum toxin antibody. k_{on} differed over 84-fold, and k_{off} differed over 33-fold, between scFv (Table 5). In vitro toxin neutralization was determined by using a mouse hemidiaphragm preparation and measuring the time to 50% twitch tension reduction for BoNT/A alone and in the presence of 2.0×10^{-8} M scFv. Values are reported in time to 50% twitch reduction. scFv binding to epitope 1 (S25) and epitope 2 (C25) significantly prolonged the time to neuroparalysis: 1.5-fold (152%) and 2.7-fold (270%), respectively (Table 5 and Fig. 3). In contrast, scFv binding to epitopes 3 and 4 had no significant effect on the time to neuroparalysis. A mixture of S25 and C25 had a significant additive effect on the time to neuroparalysis, with the time to 50% twitch reduction increasing 3.9-fold (390%).

TABLE 5. Affinities, binding kinetics, and in vitro toxin neutralization results of scFv selected from phage antibody libraries

scFv clone	Epitope	K_d[a] (M)	k_{on} (10^4 M^{-1} s^{-1})	k_{off} (10^{-3} s^{-1})	Paralysis time (min)[b]
S25	1	7.3×10^{-8}	1.1	0.82	85 ± 10[c]
C25	2	1.1×10^{-9}	30	0.33	151 ± 12[c]
C39	2	2.3×10^{-9}	14	0.32	139 ± 8.9[c]
1C6	3	2.0×10^{-8}	13	2.5	63 ± 3.3
1F3	4	1.2×10^{-8}	92	11	52 ± 1.4
C25 + S25					218 ± 22[c,d]
BoNT/A pure toxin (control)					56 ± 3.8

[a] k_{on} and k_{off} were measured by surface plasmon resonance, and K_d was calculated as k_{off}/k_{on}.

[b] Time to 50% twitch reduction in mouse hemidiaphragm assay using 20 nM scFv plus 20 pM BoNT/A compared to time for BoNT/A alone. For the C25 plus S25 combination, 20 nM each scFv was used. Each value is the mean ± standard error of the mean of at least three observations.

[c] $P < 0.01$ compared to BoNT/A.

[d] $P < 0.05$ compared to C25.

DISCUSSION

BoNTs consist of a heavy and a light chain linked by a single disulfide bond. The carboxy-terminal half of the toxin binds to a specific membrane receptor(s), resulting in internalization, while the amino-terminal half mediates translocation of the toxin from the endosome into the cytosol. The light chain is a zinc endopeptidase which cleaves an essential synaptosomal protein, leading to failure of synaptic transmission and paralysis. Effective immunotherapy must prevent binding of the toxin to the receptor, since the other two toxin functions occur intracellularly. Identification of epitopes on H_C which mediate binding is an essential first step, both to the design of better vaccines and to development of a high-titer neutralizing monoclonal antibody (or antibodies) for passive immunotherapy.

For this work, we attempted to direct the immune response to a neutralizing epitope(s) by immunization with recombinant BoNT/A H_C. This should lead to the production of antibodies which prevent binding of toxin to its cellular receptor(s). One limitation of this approach is the extent to which recombinant H_C mimics the conformation of H_C in the holotoxin. The fact that 50% of antibodies selected on H_C recognize holotoxin suggests significant structural homology for a large portion of the molecule. Although 50% of antibodies selected on H_C do not bind holotoxin, this could result from packing of a significant portion of the H_C surface against other toxin domains. Our results do not, however, exclude the possibility that some of these antibodies are binding H_C conformations that do not exist in the holotoxin or that conformational epitopes present in the holotoxin are absent from recombinant H_C. This could lead to failure to generate antibodies to certain conformational epitopes. Regardless, immunizing and selecting with H_C resulted in the isolation of a large panel of monoclonal antibodies which bind holotoxin. In contrast, monoclonal antibodies isolated after immunization with holotoxin or toxoid bind to other toxin domains (H_N or light chain) or to nontoxin proteins present in crude toxin preparations and toxoid (results from library 1, this work and reference 17).

To produce and characterize the greatest number of monoclonal antibodies possible, we used phage display. This approach makes it possible to create and screen millions of different antibodies for binding. The resulting antibody fragments are already cloned and can easily be sequenced to identify the number of unique antibodies. Expression levels in *E. coli* are

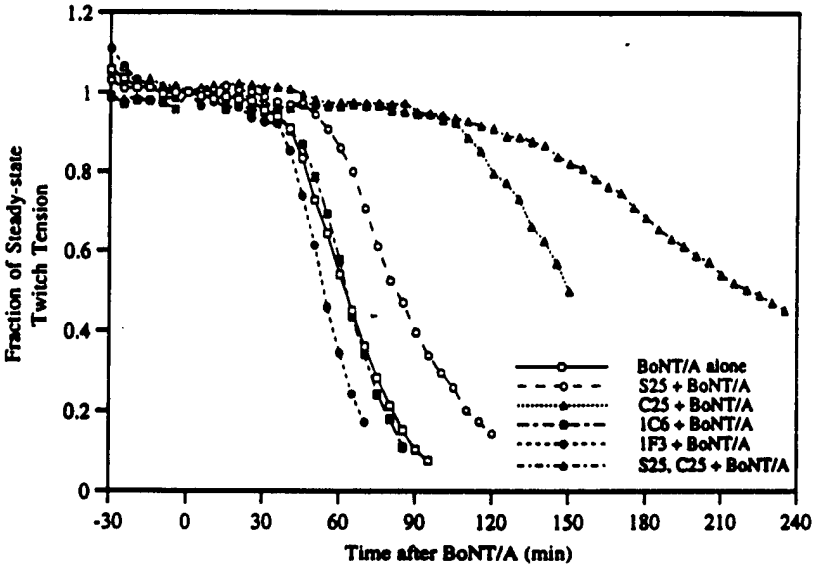

FIG. 3. Evaluation of scFv neutralization of BoNT/A in a mouse hemidiaphragm model. The twitch tension developed after electrical stimulation of a mouse hemidiaphragm was measured before (−30 to 0 min) and after the addition of 20 pM BoNT/A (control), 20 pM BoNT/A plus 20 nM scFv S25, C25, 1C6, or 1F3 (representing epitopes 1 to 4, respectively), or a combination of S25 and C25 at a final concentration of 20 nM each. Results are expressed as the fraction of steady-state twitch tension (at 0 min) versus time. scFv 1C6 and 1F3 do not alter the time to 50% twitch reduction, whereas scFv C25 and S25 significantly prolong it. The combination of S25 and C25 significantly prolonged the time to neuroparalysis compared to C25 or S25 alone.

typically adequate to produce milligram quantities of scFv, which can easily be purified by IMAC after subcloning into a vector which attaches a hexahistidine tag to the C terminus. Ultimately, the V_H and V_L genes can be subcloned to construct complete IgG molecules, grafted to construct humanized antibodies, or mutated to create ultrahigh-affinity antibodies. By this approach, 28 unique monoclonal anti-BoNT/A H_C antibodies were produced and characterized. The antibody sequences were diverse, consisting of 3 different V_H-gene families, at least 13 unique V-D-J rearrangements, and 3 V_κ-gene families. Generation of this large panel of BoNT/A H_C antibodies was a result of the choice of antigen used for immunization and selection (BoNT/A H_C). For example, a Fab phage antibody library constructed from the V genes of mice immunized with pentavalent toxoid yielded only two Fab which bound pure toxin (in this case, BoNT/B). The majority of the Fab bound nontoxin proteins present in the toxoid (17).

Despite the sequence diversity of the antibodies, epitope mapping revealed only four nonoverlapping epitopes. Epitopes 1 and 2 were immunodominant, being recognized by 21 of 28 (75%) of the antibodies. Interestingly, approximately the same number (three to five) of immunodominant BoNT/A H_C peptide (nonconformational) epitopes are recognized by mouse and human polyclonal antibodies after immunization with pentavalent toxoid and by horse polyclonal antibodies after immunization with formaldehyde-inactivated BoNT/A (3).

scFv binding epitopes 1 and 2 resulted in partial antagonism of toxin-induced neuroparalysis at the mouse neuromuscular junction. When administered together, the two scFv had an additive effect, with the time to neuroparalysis increasing significantly. These results are consistent with the presence of two unique receptor binding sites on BoNT/A H_C. While the BoNT/A receptor(s) has not been formally identified, the results are consistent with those of ligand binding studies, which also indicate two classes of receptor binding sites on toxin, high and low affinity, and have led to a "dual receptor" model for toxin binding (40). Whether both of these sites are on H_C, however, is controversial. In two studies, BoNT/A H_C partially inhibited binding and neuromuscular paralysis (4, 5), whereas Daniels-Holgate and Dolly (12) showed that BoNT/A H_C inhibited binding at motor nerve terminals but had no antagonistic effect on toxin-induced neuroparalysis at the mouse neuromuscular junction. Our results are consistent with the presence of two "productive" receptor binding sites on H_C which result in toxin internalization and toxicity. Differences in scFv potency may reflect differences in affinity of H_C for receptor binding sites or may reflect the greater than 10-fold difference in affinity of scFv for H_C. Finally, we have not formally shown that any of the scFv actually block binding of toxin to the cell surface. It is conceivable that the observed effect on time to neuroparalysis results from interference with a postbinding event.

scFv antagonism of toxin-induced neuroparalysis in the mouse hemidiaphragm assay was less than that (7.5-fold prolongation of time to neuroparalysis) observed for 2.0×10^{-9} M polyclonal equine antitoxin (PerImmune Inc.) (47a). This difference could be due to the necessity of blocking additional binding sites, differences in antibody affinity or avidity, or a cross-linking effect leading to aggregated toxin which cannot bind. Affinity of antibody binding is also likely to be an important factor, since the toxin binds with high affinity to its receptor (51) and can be concentrated inside the cell by internalization. Of note, the most potent scFv has the highest affinity for H_C. Availability of other scFv described here, which recognize the same neutralizing epitope but with different K_ds, should help define the importance of affinity. These scFv, however, differ by many amino acids and may also differ in fine specificity, making interpretation of results difficult. Alternatively, mutagenesis combined with phage display can lead to the production of scFv which differ by only a few amino acids in sequence but vary by several orders of magnitude in affinity (47). The same approach can be used to increase antibody affinity into the picomolar range (47).

The "gold standard" for neutralization is protection of mice against the lethal effects of toxin coinjected with antibody. While the relationship between in vitro and in vivo protection

has not been formally established, equine antitoxin potentially neutralizes toxin in both types of assays (see above and reference 21). Presumably, this relationship holds for the scFv reported here, but this will need to be verified experimentally. Such studies are not possible with small (25-kDa) scFv antibody fragments. The small size of scFv leads to rapid redistribution (the half-life at α phase is 2.4 to 12 min) and clearance (the half-life at β phase is 1.5 to 4 h) and antibody levels which rapidly become undetectable (26, 46), while toxin levels presumably remain high (23). Performance of in vivo studies will require the construction of complete IgG molecules from the V_H and V_L genes of scFv. Use of human constant regions will yield chimeric antibodies less immunogenic than murine monoclonals and much less immunogenic than currently used equine antitoxin. Immunogenicity could be further reduced by CDR grafting to yield humanized antibodies.

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Appendix Two

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Cell Biology

Efficient construction of a large nonimmune phage antibody library: The production of high-affinity human single-chain antibodies to protein antigens

(single-chain Fv/phage display/antibody libraries/human mAbs)

MICHAEL D. SHEETS*†‡, PETER AMERSDORFER§, RICARDA FINNERN§, PETER SARGENT¶, ERICKA LINDQVIST‖, ROBERT SCHIER§, GRETE HEMINGSEN§, CINDY WONG§, JOHN C. GERHART*, AND JAMES D. MARKS‡§

Departments of *Molecular Cell Biology and ‖Public Health, University of California, Berkeley, CA 94720; §Department of Anesthesia and Pharmaceutical Chemistry, University of California, Room 3C-38, San Francisco General Hospital, 1001 Potrero, San Francisco, CA 94110; †Department of Biomolecular Chemistry, University of Wisconsin, Madison, WI 53706; and ¶Department of Stomatology, University of California, San Francisco, CA 94143

Contributed by John C. Gerhart, March 16, 1998

ABSTRACT **A large library of phage-displayed human single-chain Fv antibodies (scFv), containing 6.7×10^9 members, was generated by improving the steps of library construction. Fourteen different protein antigens were used to affinity select antibodies from this library. A panel of specific antibodies was isolated with each antigen, and each panel contained an average of 8.7 different scFv. Measurements of antibody–antigen interactions revealed several affinities below 1 nM, comparable to affinities observed during the secondary murine immune response. In particular, four different scFv recognizing the ErbB2 protein had affinities ranging from 220 pM to 4 nM. Antibodies derived from the library proved to be useful reagents for immunoassays. For example, antibodies generated to the *Chlamydia trachomatis* elementary bodies stained *Chlamydia*-infected cells, but not uninfected cells. These results demonstrate that phage antibody libraries are ideally suited for the rapid production of panels of high-affinity mAbs to a wide variety of protein antigens. Such libraries should prove especially useful for generating reagents to study the function of gene products identified by genome projects.**

Antibodies that bind with high specificity and high affinity to a target molecule are essential tools for biological research. These reagents have proven invaluable for: (*i*) detecting and quantitating levels of gene expression; (*ii*) determining the subcellular, cellular, and tissue location of gene expression; and (*iii*) identifying the molecules interacting with a gene product, for example by immunoprecipitation.

Numerous new applications for basic research, as well as clinical use, have resulted from the development of recombinant antibodies constructed from Ig variable (V) region genes (1–3). Single-chain Fv antibodies (scFv) have proven particularly useful. scFv consist of the antigen-binding domains of Ig heavy (V_H) and light (V_L) chain regions connected by a flexible peptide linker (4), all encoded by a single gene. The single gene design of scFv simplifies the construction of fusion proteins such as cancer immmunotoxins (5) and facilitates intracellular expression in eukaryotic cells to achieve phenotypic knockout of antigen function (6–8). The intracellular expression of antibodies is proving to be an effective new strategy for studying the function of specific proteins *in vivo* where conventional genetic approaches are not feasible.

Genome projects have led to an increasing rate of gene discovery and an accelerating need for antibodies to study gene expression and function. Until recently, hybridoma technology, a slow and cumbersome process, was used to produce mAbs for such applications. Separate immunizations are required for each antigen, and the cell fusion process required to generate hybridomas is laborious and inefficient. In addition, production of antibodies to antigens conserved between species is difficult and antibodies from hybridomas are murine and hence immunogenic if used therapeutically.

Recent advances using antibody phage display now make it possible to overcome these limitations and generate human mAbs that recognize any desired antigen (1–3, 9). For phage display, the antigen-binding regions of V_H and V_L genes are cloned and used to construct scFv (or Fab) gene repertoires. A phage antibody library is created by cloning these repertoires as fusion proteins with a minor coat protein of bacteriophage (the gene 3 protein) (10–12). Each resulting phage has a functional antibody protein on its surface and contains the gene encoding the antibody incorporated into the phage genome. Particular phage antibodies that specifically bind to proteins and small molecules can be separated from nonbinding phage antibodies with affinity chromatography techniques (12–15). This strategy requires no immunization, the antibody genes are cloned, and generally the antibody fragments express well in *Escherichia coli*. The number and affinity of the antibodies generated to a particular antigen is a function of library size and diversity, with larger libraries yielding a greater number of high-affinity antibodies (14, 15). Unfortunately, the construction of large phage-displayed antibody libraries has remained difficult. If such libraries are to be a common tool of life scientists the efficient production of these reagents must become routine, especially because library diversity and utility are lost on library reamplification.

In this paper, we describe a strategy to optimize the construction of phage-display antibody libraries. By using this strategy, a very large phage-displayed single-chain antibody library consisting of 6.7×10^9 members was produced. This library then was used to isolate panels of antibodies to 14 different protein antigens. Analysis of antibody–antigen interactions revealed high-affinity binding with K_ds for the ErbB2 protein ranging between 220 pM and 4 nM.

METHODS

Construction of the V_H Library. Total RNA was prepared from three different samples of human spleen cells and two

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Abbreviations: BMP, bone morphogenetic protein; BoNT, botulinum neurotoxin; ECD, extracellular domain; CDR, complementarity determining region; EB, elementary body; scFv, single-chain Fv fragment; V_κ, Ig kappa light chain variable region; V_λ, Ig lambda light chain variable region; V_L, Ig light chain variable region; V_H, Ig heavy chain variable region.

‡To whom reprint requests should be addressed.

different samples of human peripheral blood lymphocytes. cDNA was synthesized from total RNA primed with the HuIgMFOR primer (12). V_H gene repertoires were amplified from the cDNA by using Vent DNA polymerase (New England Biolabs) in combination with the HuIgMFOR primer and an equimolar mixture of HuVHBACK primers (12). PCR products were agarose gel-purified and reamplified to append *Nco*I and *Not*I restriction sites by using Tth DNA polymerase (Epicentre Technologies, Madison, WI) and an equimolar mixture of the HuVHBACKSfi primers (that contain an *Nco*I site for cloning) and the HuCMForNot primer (5′-GAGTC-ATTCTCGACTTGCGGCCGCTGGAAGAGGCACGTTC-TTTTCTTT-3′) (12). The PCR products were cut with restriction enzymes *Nco*I and *Not*I and agarose gel-purified. The resulting DNA fragments were ligated into the plasmid pCITE3A (Novagen) cut with restriction enzymes *Nco*I and *Not*I and the ligated DNA was electroporated into the *E. coli* strain TG1. A library of V_H genes containing 2.3×10^8 members was generated from the products of seven ligation reactions and 15 electroporations. The resulting library was termed pCITE-V_H. Cloning efficiency and library diversity was determined by PCR screening (12, 16). The pCITE3A plasmid was used to create the V_H gene repertoire because of the presence of unique sequences for PCR amplification that surround the *Nco*I and *Not*I cloning sites. These sequences allow the specific amplification of the V_H genes for scFv assembly. This strategy is advantageous for amplification of the V_H genes and also the subsequent amplification of scFv genes assembled from the V_H genes. Although we chose the pCITE3A plasmid for production of our V_H gene repertoire, any plasmid that contains the proper restriction sites for cloning and unique sequences for specific PCR amplification would have been suitable.

Construction of the scFv Library. The V_H gene repertoire was PCR-amplified from the pCITE-V_H library by using 300 ng of library plasmid DNA as a template, Vent DNA polymerase, the CITE3 primer (5′-GATCTGATCTGGGGCCTCGGT-GC-3′), and an equimolar mixture of HuJ_H primers (12). The V_L genes for scFv assembly were obtained from a previously constructed scFv phage antibody library (12). The V_L gene repertoire, including DNA encoding the scFv peptide linker $(G_4S)_3$ (4), was amplified from 300 ng of library plasmid DNA by using Vent DNA polymerase, the Gene3 primer (5′-GC-AAGCCCAATAGGAACCCATGTACCG-3′), and an equimolar mixture of $RHuJ_H$ primers (12). The amplified V_H and V_L genes were agarose gel-purified and spliced together with overlap extension PCR to create a scFv gene repertoire (11). To accurately join V_H and V_L gene repertoires with overlap extension PCR, the input DNA fragments must have blunt ends. Therefore, the proofreading DNA polymerase Vent was used to generate the V_H and V_L DNA fragments for scFv assembly. For all subsequent PCR steps of library construction Tth DNA polymerase was found to be the optimal enzyme. The V_H and V_L gene repertoires were spliced together in 100- μl PCRs containing 100 ng of the V_H and V_L DNA fragments and Tth DNA polymerase. The reactions were cycled eight times (95°C 2 min, 55°C, 1 min, and 72°C 3 min) to join the fragments. Then the CITE3 and Gene3 primers were added and the reaction was cycled 30 times (94°C 1 min, 55°C 1 min, and 72°C 3 min) to amplify the assembled scFv genes. The scFv genes were cut with restriction enzymes *Nco*I and *Not*I, agarose gel-purified, and ligated into the plasmid pHEN-1 (17) cut with *Nco*I and *Not*I. The ligated DNA was electroporated into *E. coli* TG1 cells.

Proteins. The extracellular domains of the *Xenopus* activin receptor type I (A. Suzuki and N. Ueno, personal communication), activin receptor type II (18), bone morphogenetic protein (BMP) receptor type I (19, 20), and fibroblast growth factor receptor (21) were cloned into pMAL expression plasmids as fusions with the gene encoding maltose binding protein expressed and purified from *E. coli.* (New England Biolabs). Neuronal bungarotoxin was purchased from Biotoxins. *Clostridia botulinum* neurotoxin type A (BoNT/A) was provided by Ray Stevens (Univ. of California, Berkeley), and BoNT/B, C, and E were provided by Theresa Smith (United States Army Medical Research Institute of Infectious Disease). BoNT/A C-fragment was purchased from Ophidian (Madison, WI). Human ErbB-2 extracellular domain (ECD) was provided by James Huston (Creative Biomolecules) (22), human cytochrome b5 was provided by by Lucy Waskell (Univ. of California, San Franscisco), and human vascular endothelial growth factor was provided by James Hoeffler (Invitrogen).

Selection of Phage Antibodies. Phagemid particles were rescued from the library, as described (23) except that the procedure was scaled up to 2 liters of culture media. Specific phage-displayed scFv were affinity-selected by using proteins absorbed to Immunotubes (Nunc) (12). For selections with maltose binding protein (MBP) fusion proteins, phage were preincubated with 50 μg of purified MBP to deplete the library of MBP antibodies. For selection of scFv to the Erb-B2 ECD, Immunotube selection was alternated with selection using decreasing concentrations of biotinylated Erb-B2 ECD and

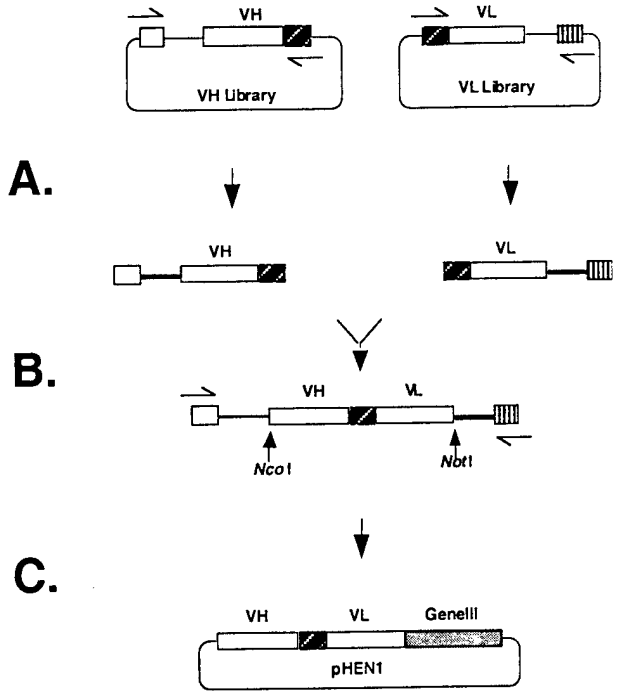

FIG. 1. Schematic outline of the approach used for library construction. A library of V_H and genes was generated from rearranged human V-genes and cloned into the plasmid pCITE3A. The V_L genes used for scFv assembly were derived from a previously constructed scFv library contained in the plasmid pHEN1 (12). The vector containing the V_L repertoire also contained the scFv linker DNA 5′ to the V_L genes. Primers for reamplification of the V-gene repertoires were derived from sequences several hundred bp 5′ (the V_H genes) or 3′ (the V_L genes) of the scFv gene cloning sites. This approach facilitated the efficiency of PCR assembling a new scFv repertoire and increasing the efficiency of cutting assembled scFv genes with restriction enzymes. (*A*) V_H and linker-V_L gene repertoires were generated by PCR from the plasmid DNA of the separate libraries. The V_H genes wereamplified by using a plasmid specific primer ( ) and an equimolar mixture of HuJ_H primers ( ). The linker DNA and V_L genes were amplified by using a plasmid specific primer ( )and an equimolar mixture of $RHuJ_H$ primers ( ). The RHuJH primers are complementary to the HuJ_H primers. (*B*) The V_H and linker DNA-V_L gene repertoires were PCR assembled into a scFv gene repertoire. (*C*) The assembled scFv gene repertoire was cut with the restriction enzymes *Nco*I and *Not*I and cloned into the plasmid pHEN1 (17) for phage display.

capture of bound phage using streptavidin paramagnetic beads (23). For selection of scFv that bind *Chlamydia* antigens, Immunotubes were coated overnight at room temperature with 1 ml of *C. trachomatis* strain L2/434/Bu elementary bodies (EBs) at a concentration of 0.1 mg/ml (in PBS) purified from a suspension culture of L929 cells (24). Phage eluted from each selection were used to infect *E. coli* TG1 cells. Phage particles were rescued from the cells and used for the subsequent round of antigen selection. The rescue-selection-plating cycle was repeated 3–4 times, after which individual clones were analyzed for specific antigen binding by ELISA.

Antibody Binding Specificity. The binding specificity of all scFv was determined by ELISA using the target antigen and at least nine other proteins as substrates (12). The number of unique scFv was estimated by PCR fingerprinting of the scFv genes with the restriction enzyme *Bst*NI and confirmed by DNA sequencing (12, 16). Putative V_H and V_L germ-line gene segment derivation was determined with the VBASE sequence directory (25).

scFv Purification and Affinity Measurements. For purification, scFv genes were subcloned, expressed, and purified to homogeneity (26). scFv dissociation equilibrium constants (K_d) were calculated from the association (k_{on}) and dissociation (k_{off}) rate constants determined by using surface plasmon resonance in a BIAcore (23, 27).

Fluorescent Cell Staining. Monolayers of HeLa 229 cells were grown on coverslips in 24-well cell culture plates. Two hundred microliters of *C. trachomatis* EBs at 8×10^6 inclusion forming units/μl were used to infect the monolayers (28). The infected cells were incubated for 48 hr at 37°C, washed with PBS, and fixed with 100% methanol for 10 min. Purified scFv (50 μg/ml) was incubated with fixed cells for 1 hr at room temperature. scFv binding was detected with the 9E10 mAb that recognizes the c-myc epitope present in the scFv (29) (1 μg/ml) followed by fluorescein isothiocyanate-conjugated anti-mouse Fc (Zymed). Cells were counterstained with Evans blue and visualized with fluorescence microscopy.

RESULTS

Library Construction. A very large phage antibody library was created for the routine isolation of high-affinity scFv antibodies to any target protein. This library was generated by optimizing the individual steps of library construction to increase the efficiency of scFv gene assembly and increase the efficiency of cloning scFv genes (Fig. 1). First, scFv antibodies were assembled from cloned V_H and V_L gene repertoires contained in separate plasmid vectors. A library of V_H genes, containing 2.3×10^8 members, was specifically created for generating an additional scFv repertoire. The V_L genes for scFv assembly were derived from an existing scFv repertoire containing 3.0×10^7 members (Fig. 1*A*). The use of cloned libraries as a source of V-genes provided a stable and limitless supply of material for scFv assembly. For the construction of previous antibody libraries, scFv gene repertoires were directly assembled from V_H and V_L reverse transcription–PCR (RT-PCR) products (12). With this previous approach, RNA availability and the efficiency of RT-PCR limited the quantity of V-genes available for scFv construction. Second, the efficiency of scFv assembly was increased by exploiting the presence of the DNA encoding the peptide $(G_4S)_3$ linker located at the 5′ end of the V_L library (Fig. 1*B*). Using V_L genes already fused to the peptide linker allowed us to construct scFv from only two DNA fragments. Previously, scFv gene repertoires were inefficiently assembled from three separate DNA fragments consisting of V_H and V_L gene repertoires and linker DNA (12). Third, the V_H and V_L gene repertiores and the scFv genes assembled from these repertoires were amplified with primers that annealed to sequences approximately 200 bp 5′ of the V_H genes and to sequences approximately 200 bp 3′ of the V_L genes. This strategy generated long sequence extensions at the ends of the individual V_H, V_L gene segments, and the assembled scFv. These sequence extensions ensured efficient cutting with the restriction enzymes *Nco*I and *Not*I that were used for scFv cloning and facilitated the identification of the correctly assembled scFv (Fig. 1*C*).

By using these three modifications a repertoire of scFv genes was efficiently assembled and cloned to create a phage antibody library containing 6.7×10^9 members. This library was generated from the products of only 12 ligation reactions and 36 electroporations. DNA sequencing of the V-genes from 36

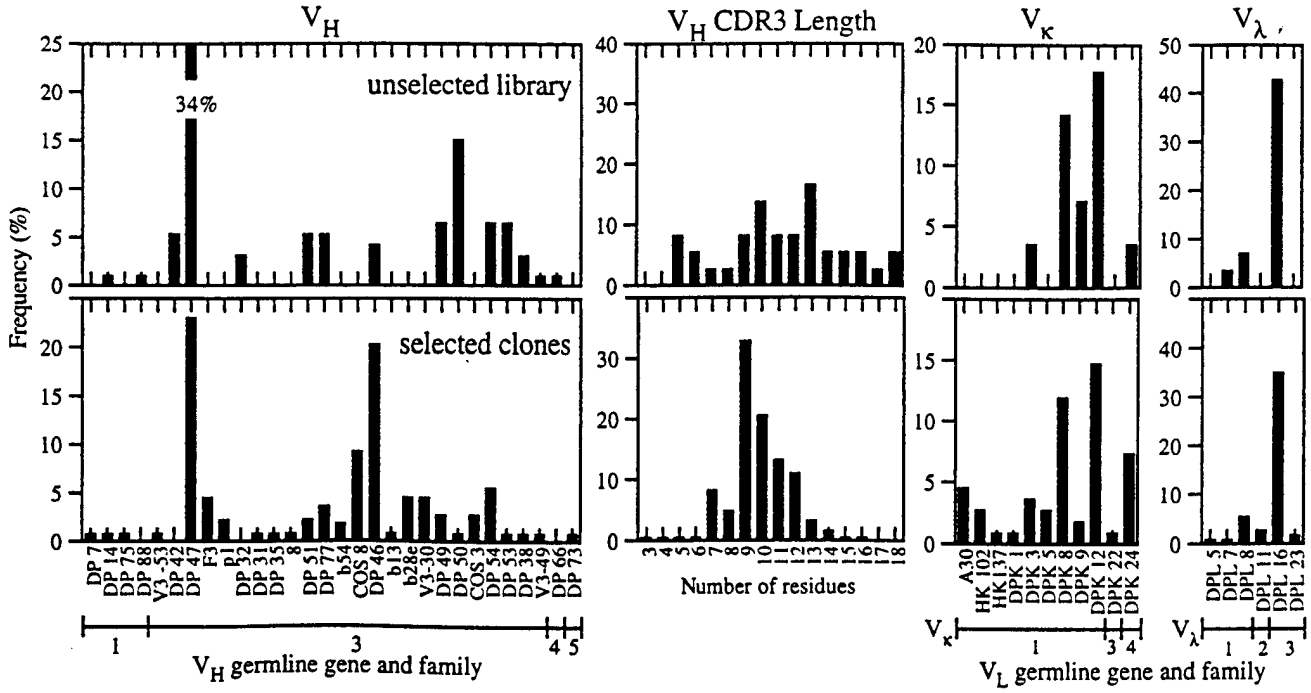

FIG. 2. V-gene usage and V_H CDR3 length of unselected and antigen-specific scFv. The V_H and V_L genes were sequenced and the germ-line gene was assigned based on homology to a database (VBASE) of germ-line V-genes compiled by Tomlinson *et al.* (25). Specific V_H, V_κ, and V_λ genes are listed on the ordinate, with the V_H, V_κ, or V_λ germ-line gene family indicated below. Only V-genes in unselected or selected clones are listed.

Table 1. Results of phage antibody library selections

Protein antigen used for selection	Percentage (number) of ELISA positive clones	Number of different antibodies isolated
FGF receptor ECD	69 (18/26)	15
BMP receptor type I ECD	50 (12/24)	12
Activin receptor type I ECD	66 (16/24)	7
Activin receptor type II ECD	66 (16/24)	4
Erb-B2 ECD	91 (31/34)	14
VEGF	50 (48/96)	6
BoNT/A	28 (26/92)	14
BoNT-A C-fragment	95 (87/92)	10
BoNT/B	10 (9/92)	5
BoNT/C	12 (11/92)	5
BoNT/E	9 (8/92)	3
Bungarotoxin	67 (64/96)	15
Cytochrome b5	55 (53/96)	5
C. trachomatis EB	66 (63/96)	7

For each antigen (column 1), the number and the percentage of positive clones selected (column 2) and the number of different antibodies isolated (column 3) is indicated. FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor.

randomly chosen scFv revealed 36 unique sequences and a relatively random distribution of V_H complementarity determining region (CDR) 3 length of between 5 and 18 residues (Fig. 2). There was, however, bias in V-gene usage, with both over-representation of specific V-gene families (V_H3, $V_\kappa1$, and $V_\lambda3$) and V-genes (DP-47, DPL 16) (Fig. 2). This bias partially reflects differential V-gene usage observed in the human B-cell repertoire (30–33) but also may be caused by differences in PCR primer annealing to the different V-genes. Previous work indicates that more diverse repertoires could be created by using V_H and V_κ gene family specific primers individually rather than pooled for construction of the V-gene repertoires (34).

Selection and Characterization of Antigen-Specific scFv. Antibodies from the phage antibody library were affinity-selected by using 13 different purified protein antigens from a variety of species, including human and EBs from *C. trachomatis* (Table 1). Given our interest in developmental biology, four of these proteins were the extracellular domains of different *Xenopus* growth factor receptors: the activin receptor types I and II, the BMP receptor type I, and the fibroblast growth factor receptor (19–21). After at least three rounds of selection with a particular antigen, the binding specificity of individual scFv was determined by ELISA. A high percentage of the clones analyzed specifically bound the antigen used for selection (Table 1, second column). To determine the number of different scFv that recognized each antigen, ELISA-positive clones first were characterized by DNA fingerprinting (12, 16) and then DNA sequencing (23). This analysis revealed an average of 8.7 different antibodies were generated to each protein antigen, with the number of scFv ranging from 3 to 15 (Table 1). Because only a small number of clones from each selection were analyzed, it is likely that screening of more clones would yield additional antibodies.

The binding of scFv to antigens was highly specific. For example, serotype specific scFv were isolated against each of the four different types of BoNT, despite 32–59% sequence homology between the toxins (Fig. 3). Another example of scFv specificity is shown in Fig. 4, where a *C. trachomatis*-specific scFv stains *C. trachomatis* elementary bodies within infected cells while neighboring uninfected cells remain unstained.

V-gene derivation of scFv antibodies that bound to the different antigens was diverse (Fig. 2). V_H genes were derived from three of the six V_H gene families (nos. 1, 3, and 5) and

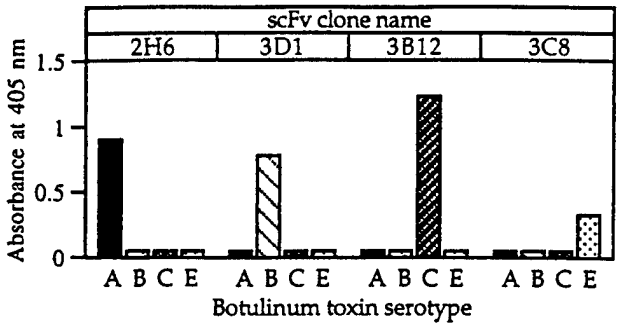

FIG. 3. Specificity of anti-Botulinum neurotoxin scFv. Representative scFv (2H6, 3D1, 3B12, and 3C8) isolated respectively from selections on BoNT serotypes A, B, C, and E were studied. Specificity was determined by ELISA.

from 26 different germ-line genes. V_L genes were derived from three of the six V_κ gene families (nos. 1, 3, and 4) and 11 different V_κ germ-line genes, from three of the nine V_λ gene families (nos. 1–3), and nine different V_λ germ-line genes. Despite the diversity, there was a bias seen in the V-gene usage. V_H genes largely were derived from the V_H3 family, particularly DP46 and DP47. V_κ genes most frequently were derived from the $V_\kappa1$ family while V_λ genes most frequently were derived from the $V_\lambda3$ family, especially DPL-16. This bias partially reflects the greater frequency of certain V-genes in the B-cell repertoire (30–33) and also in the unselected library (for example DP-47 and DPL-16). Differential V-gene usage also may reflect expression biases of *E. coli*. The number of sequenced V-genes from previous nonimmune phage antibody libraries is small (approximately 30) but a similar bias in V-gene usage is observed (12, 35, 36). V_H CDR length of selected clones was not as evenly distributed as in the unselected clones (Fig. 2) with the majority of lengths between 7 and 15 amino acids. A similar peak is seen in V_H CDR3 length of antibodies generated *in vivo* (37).

Affinity of Selected Antibodies. The antibody-antigen binding affinities were measured for several of the anti-ErbB-2 and anti-BoNT/A scFv. The genes of four anti-ErbB-2 scFv and four anti-BoNT/A scFv were subcloned into a plasmid to add a hexahistidine tag, then expressed and purified from *E. coli*. The dissociation equilibrium constants (K_d) of purified soluble anti-ErbB-2 and anti-BoNT/A scFv were calculated from association and dissociation rate constants measured by using surface plasmon resonance (Table 2) (23, 27). The K_d of the antibodies ranged from 220 pM to 4 nM for anti-ErbB-2 scFv and 38 nM to 71 nM for anti-BoNT/A scFv. The affinity of the

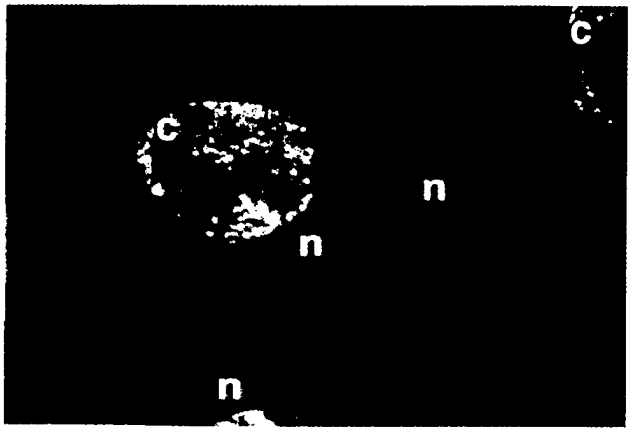

FIG. 4. Staining of HeLa cells infected with *C. trachomatis* with the scFv 2A10. The scFv specifically stains *C. trachomatis* elementary bodies (c) within infected HeLa cells but does not stain uninfected cells. n = nucleus.

Table 2. Affinities and binding kinetics of anti-BoNT A C-fragment and anti-Erb-B2 scFv

Specificity and clone	K_d ($\times 10^{-9}$ M)	k_{on} ($\times 10^5$ $M^{-1}s^{-1}$)	k_{off} ($\times 10^{-3}$ s^{-1})
ErbB-2 B7A	0.22	4.42	0.1
ErbB-2 G11D	0.48	2.19	0.11
ErbB-2 A11A	0.49	3.69	0.18
ErbB-2 F5A	4.03	1.62	0.65
BoNT-A 2A9	26.1	0.25	0.66
BoNT-A 2H6	38.6	2.2	8.5
BoNT-A 3F6	66.0	4.7	30.9
BoNT-A 2B6	71.5	1.1	7.8

Association (k_{on}) and dissociation (k_{off}) rate constants for purified scFv were measured by using surface plasmon resonance (BIAcore) and K_d was calculated as (k_{off}/k_{on}).

anti-ErbB-2 scFv B7A is the highest observed for any antibodies isolated from nonimmune phage antibody libraries (14, 15). The affinities of the isolated scFv are also comparable to affinities of mAbs derived from the secondary immune response (38).

The different K_ds observed for scFv that bind ErbB-2 and BoNT/A are probably a consequence of the different selection conditions used to isolate each panel of antibodies. ErbB-2 antibodies were selected with decreasing concentrations of soluble antigen captured with magnetic beads alternated with selections using immobilized antigen. The use of soluble antigen is a more efficient method for controlling the concentration of antigen used for selection and isolating scFv with higher affinity (23, 39). Therefore, reselection of antibodies using decreasing concentrations of BoNT/A would likely lead to the isolation of antibodies with higher binding affinity.

DISCUSSION

A very large scFv phage antibody library was efficiently generated and its use as a resource for the production of antibodies was extensively evaluated. By using a number of different criteria, the results validate our methods for constructing large libraries of this type and validate the use of these libraries as a resource for the rapid production of antibodies (Table 1). First, by using 14 different proteins for affinity selection, specific antibodies were successfully generated to each of these antigens (Table 1). Second, a high percentage of the antibodies that resulted from affinity selection specifically recognize antigen (Table 1). Third, multiple different antibodies were produced to each antigen (Table 1). Fourth, the binding affinities of the antibodies isolated were comparable to those of mAbs from the secondary murine immune response (Table 2). In addition, these antibody antigen binding affinities are the highest reported for antibodies from nonimmune phage antibody libraries (12, 14, 15). Fifth, isolated scFv served as functional reagents in a number of different immunoassays including ELISA, immunofluorescence (Figs. 3 and 4), Western blotting, epitope mapping, and immunoprecipitation (data not shown).

Nonimmune phage antibody libraries can be constructed as either scFv or Fab antibody fragments and from either V-genes rearranged *in vivo* or synthesized *in vitro*. The scFv format was chosen for this library as the expression levels in *E. coli* are typically higher than Fab. This results in more efficient antibody display on phage and more efficient production of native antibody fragments for use. V-genes rearranged *in vivo* were used for library construction to eliminate the need for cloning the individual gene segments necessary for *in vitro* V-gene synthesis. In addition, use of Ig mRNA as the source of V-genes ensures that close to 100% of the gene sequences will be functionally rearranged with ORFs (results from this work and ref. 34). Fewer V-genes will have an ORF when constructed from synthetic oligonucleotides. Furthermore, V-genes rearranged *in vivo* have V_H CDR3s largely derived from the D-gene segments. These genes are not of random sequence but encode amino acids with a propensity for loop formation (40). In contrast, synthetic CDR3s consist of random sequence and thus may be less likely to fold properly or produce usefully shaped binding pockets.

The number and affinities of antibodies produced from this library compare favorably to results from the limited number of phage antibody libraries previously described (Table 3). A comparison of nonimmune libraries illustrates the importance of library size and also suggests that to date, the most useful libraries are those in the scFv format constructed from V-genes rearranged *in vivo*.

Nonimmune phage antibody libraries already are being used as a source of diagnostic and therapeutic antibodies. It is likely that their greatest utility, however, may lie in the laboratory. New genes are rapidly being identified by the genome projects, and the next generation of experiments will shift to elucidating the function of the protein products encoded by these newly identified genes (41). The production of antibodies with phage-displayed libraries is ideally suited for the large-scale determination of protein function. For example, once a gene has been sequenced, the protein(s) that it encodes can be overexpressed and then used to rapidly select phage-displayed antibodies. The resulting antibodies would provide immunological reagents for protein characterization. In addition, the production of antibodies with phage display also provides access to the genes that encode specific antibodies. These antibody genes can be used to express antibody proteins within cells to block and elucidate the function of specific molecules *in vivo* (6–8).

In summary, the steps of phage antibody library construction have been optimized to facilitate the rapid and efficient construction of large phage antibody libraries. With this current library we obtain panels of high-affinity antibodies to a wide array of antigens. The approach used puts this technique within reach of laboratories skilled in molecular biology. Subsequent uses for these libraries will be limited only by the investigator's imagination.

Table 3. Comparison of protein binding antibodies selected from nonimmune phage-display antibody libraries

Library	Library size and type*	Number of protein antigens studied	Average number of antibodies per protein antigen	Number of affinities measured	Range of affinities for protein antigens K_d ($\times 10^{-9}$ M)
Marks *et al.* (12)	3.0×10^7 (scFv, N)	2	2.5	1	100–2000
Nissim *et al.* (13)	1.0×10^8 (scFv,SS)	15	2.6	ND	ND
deKruif *et al.* (42)	3.6×10^8 (scFv,SS)	12	1.9	3	100–2,500
Griffiths *et al.* (14)	6.5×10^{10}(Fab, SS)	30	4.8	3	7.0–58
Vaughan *et al.* (15)	1.4×10^{10}(scFv, N)	3	7.0	3	4.2–8.0
Sheets *et al.* (this work)	6.7×10^9 (scFv, N)	14	8.7	8	0.22–71.5

*For library type, N = V-gene repertoires obtained from V-genes rearranged *in vivo*; SS = semisynthetic V-genes constructed from cloned V-gene segments and synthetic oligonucleotides encoding V_H CDR3. ND, not determined.

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Appendix Three

Table 10: Partial V_H sequences, specificities, and germline gene assignment of α-BoNT/A scFv from a human immune phage antibody library.

Bind BoNT/A but not C-fragment or translocation domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH3DP47	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
cl3C6	----------	-----	----------	T-----DT-------E-	----------N-E----G----------	FGRPDH	W
cl3E9	-R--------S----------	-N--T	-G---G--------	----------	----------S----------F---	APNIFPAYFDY	WGQ
cl3H4[a]	-R--------S----------	-N--T	-G---G--------	----------	----------S----------F---	APNIFPAYFDY	WGQ
VH3DP51	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYSMN	WVRQAPGKGLEWVS	YISSSSSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRDEDTAVYYCAR		
cl3H3	T----I-N	NFG--	-------R------	---GD-D----P---R-	----------D---D----D----------	DTGYKRLSSTWAFDL	WG
cl3B12	T----I-N	NFG--	-------R------	---GD-D----P---R-	----------D---D----D----------	DTGYKRLSSTWAFDL	WG
VH4DP66	QVQLQESGPGLVKPSETLSLTCTVSGGSVS	SYYWS	WIRQPPGKGLEWIG	YIYYSGSTNYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR		
cl3D2	-----	-----	----------	----------	----------	DPRGRSGYYSDAFDI	WGQ
cl3D3			---TH-H-------	IF----------	----------	DPRGRSGYYSDAFDI	WGQ
cl3E11		-----	----S---------	--S---T-E-------	--T----------	AASLSCTGSSCRYNYFDP	WG
VH3DP46	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
cl4E4	----------RV-	-F---	----------M-	-L-H--N-I--------	--I----------L----------	GFEHRGVVVMYYYNAMDV	WGQ
cl3A2		-F--T	----------M-	-L-H--N-I--------	--I----------L----------	GFEHRGVVVMYYYNAMDV	W
cl3E3[b]		-F--T	----------M-	-L-H--N-I--------	--I----------L----------	GFEHRGVVVMYYYNAMDV	W
cl3E7		-F---	----------M-	-L-H--N-I--------	--I----------L----------	GFEHRGVVVMYYYNAMDV	W
cl3A1[c]		-F---	----------M-	-L-H--N-I--------	--I----------L----------	GFEHRGVVVMYYYNAMDV	W
cl4B4[d]		-F---	----------M-	-L-H--N-I--------	--I----------L----------	GFEHRGVVVMYYYNAMDV	W
cl3E8	---------- VT	-FLYV	----------VR--	SFALVG-IY-------	--I----------L----------	GFEHRGVVVMYYYNAMDV	WGQG
Bind C-fragment and BoNT/A							
VH1DP10	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS	SYAIS	WVRQAPGQGLEWMG	GIIPIFGTANYAQKFQG	RVTITADESTSTAYMELSSLRSEDTAVYYCAR		
cl2B11	-------D--R	--I--	----------	----M---T----Q---	K------D------L--R--T----------	GPWELVGYFDS	WG
VH1V1-2	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	GYYMH	WVRQAPGQGLEWMG	WINPNSGGTNYAQKFQG	RVTMTRDTSISTAYMELSRLRSDDTAVYYCAR		
cl3F10	----------R--------A--	A----	----------	R------D--S------	----------N-------S---------F-V-	LATYYYFGLDV	WGQ
cl3B8[e]	----------R--------A--	A----	----------	R------D--S------	----------N-------S---------F-V-	LATYYYFGLDV	WGQ
VH3DP50	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	VIWYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
cl3D12			----	---F--TE-IS-E----	----------F--------D----------	EPDWLLWGDRGALDV	W
cl3A6	--------GV----M	--SID	----------	-M-F--TE--S-E----	-----S-------F------S-D--------S	EPDWLLWGDSGALDV	WGQ
VH1DP2	QMQLVQSGPEVKKPGTSVKVSCKASGFTFT	SSAVQ	WVRQARGQRLEWIG	WIVVGSGNTNYAQKFQE	RVTITRDMSTSTAYMELSSLRSEDTAVYYCAA		
cl2B10	-S-S	NY---	----------	-T-----PSK------D	--------A-N------N--T-------F---	GRVWTYYYGSRSLEGFDI	WGQ
VH3DP46	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
cl2C7	----	-H---	----------	----------	----------L-----	LAGGYDYRASSYYYHHHMDV	WG
Bind translocation domain and BoNT/A							
VH3DP54	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYWMS	WVRQAPGKGLEWVA	NIKQDGSEKYYVDSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR		
cl3D4	----------	----N	--P-F--R------	S--H-A--R--------	----------L----V---------K	GRQWLVQSYFES	WG
cl3A11[f]	----------	----N	--P-F--R------	S--H-A--R--------	----------L----V---------K	GRQWLVQSYFES	WG
cl4A4	----------	----N	----------	S--H-A--R--------	----------L----V---------K	GRQWLVQSYFES	WGQG
VH3DP51	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYSMN	WVRQAPGKGLEWVS	YISSSSSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRDEDTAVYYCAR		
cl3G4			----------	------DL------A--	----------F----------	ERFRELFHLLGY	W

a. Same V_H gene as cl3E9, but different light chain, b. Same V_H gene as cl3A2, but different light chain, c. Same V_H gene as cl3E7 and cl4B4, but different light chain, d. Same V_H gene as cl3E7 and cl3A1, but different light chain, e. Same V_H gene as cl3F10, but different light chain, f. Same V_H gene as cl3D4, but different light chain

Table 11: Partial V_L sequences, specificities, and germline gene assignment of α-BoNT/A scFv from a human immune phage antibody library.

Bind BoNT/A but not C-fragment or translocation domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VkI DPK8	DIQLTQSPSFLSASVGDRVTITC	RASQGISSYLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	QQLNSYP	
cl3A1	-------	W-GT--NN---	-I--Q-----R----	-------	----------R----------F-	--VD---LT	FGGGTK
cl3E3[a]	-------	W-GT--NN---	-I--Q-----R----	-------	----------R----------F-	--VD---LT	FGGGTK
cl4E4	-------	W-G---NN---	-----Q-----R----	-------	----------R----------F-	--VD---LT	FGGG
cl3A2[b]	-------	W-G---NN---	-----Q-----R----	-------	----------R----------F-	--VD---LT	FGGG
cl3E7	-------	W-G-A-NN---	-----Q-----R----	-------	----------R----------F-	--VD---LT	FGGG
cl3E8		--	-----Q-A-------L	-------	----------R----------F-	--VD---LT	FGGGTK
VKI DPK9	DIQMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP	
cl3C6	----------	----------	-----S-----N----	-------	----------N----------	-----DA-YT	FGQGTK
cl3H3	-----T--	----------	----------F----	-----S--	-A----------	-----T--RT	FGQGTK
cl3B12		---N-RNS--	-----Q---------	T-TN----	----------F--E----------D--------	--YH-Y-YT	FGQGTK
VKI L12[2]	DIQMTQSPSTLSASVGDRVTITC	RASQSISSWLA	WYQQKPGKAPKLLIY	KASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYNSYS	
cl4B4		-----R-NN---	-----Q-------F---	NT------	----------	--V---PRWT	FGQGTK
cl3E9	-------	-----R-NN---	----------F----	-T------	----------	-------PWT	FGQ
cl3D2		---N-RNS-N	-----Q---------	T-TN-Q--	----------	---H--PYT	F
cl3H4	----------	-----R-NN---	----------F----	-T------	----------	-------PWT	FGQ
Bind C-fragment and BoNT/A							
VkI DPK8	DIQLTQSPSFLSASVGDRVTITC	RASQGISSYIA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	QQLNSYP	
cl3F10	-----	---S-----N--	----------	N-------	-----S----------F-	--------LT	FGG
VKI DPK7	DIQMTQSPSSLSASVGDRVTITC	RASQGISSWLA	WYQQKPEKAPKSLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQYNSYP	
cl3B8	----------	- -----R---	-------G-----L---	D--T----	----------N-----D--------	-------VYT	FG
VKI L12[2]	DIQMTQSPSTLSASVGDRVTITC	RASQSISSWLA	WYQQKPGKAPKLLIY	KASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYNSYS	
cl2B11		----------	----------M-	E-------	----------A----	-H--T-PYT	FGQGTK
cl3A6[c]		----------	----------M-	E-------	----------A----	-H--T-PYT	FGQGTK
cl3D12	----------	-----G------	----------M-	E-------	----------A----	-H--T-PYT	FGQGT
Bind translocation domain and BoNT/A							
VKIV DPK24	DIVMTQSPDSLAVSLGERATINC	KSSQSVLYSSNNKNYLA	WYQQKPGQPPKLLIY	WASTRES	GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC	QQYYSTP	
cl3D4	------	-------C----------	----------	-------	----------T----------	--------PT	FGGGTK
cl3A11	------	----------	----------	-------	----------RP----------	--------PT	FGGGTK
cl4A4		------I--	-I----------	-------	----------RP----------	--------PT	FGGGTK
cl3G4	------	----------	----------	-------	----------RP----------	--------LT	FGGGTKV

a. Same V_k gene as cl3A1, but different heavy chain
b. Same V_k gene as cl4E4, but different heavy chain
c. Same V_k gene as cl2B11, but different heavy chain

Table 12. Partial V_H and V_L sequences and germline gene assignment of α-BoNT/B scFv from a human immune phage antibody library.

V_H Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH1DP10	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS	SYAIS	WVRQAPGQGLEWMG	GIIPIFGTANYAQKFQG	RVTITADESTSTAYMELSSLRSEDTAVYYCAR		
cl2D4, 2D12	----q-------G----------S---S-T	--S-H	----------A	M---M---S--------	----------G----------K	TPQHFYYYAMDV	WGQ
VH1V1-2	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	GYYMH	WVRQAPGQGLEWMG	WINPNSGGTNYAQKFQG	RVTMTRDTSISTAYMELSRLRSDDTAVYYCAR		
2D1	--------P----------	--N-Y	----------	R----------	--S------M--------S----------	EWTQLWSPYDY	WG
3A9	----q-------S-----N---------I	---IY	----------	-------V-K-------	------ ------N---A----------	VDTVMVPSMII	WGQ
cl2A2, 2B5	----q--------L-L--N----------	---IY	----------	------N-V-K-------	-----I----------N----------	EWTQLWSPYDY	WG
VH3DP77	EVQLVESGGGLVKPGGSLRLSCAASGFTFS	SYSMN	WVRQAPGKGLEWVS	SISSSSSYIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR		
cl2E1	q----q----------T---A--	NFIVA	----------	--TGP-H--------R-	---------R----------	GRLPAYHFDY	WGQG
VH3DP49	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
cl2G1	----qq----------V------R	TF---	----------	F--S--GT---V---E-	---------R--VFV-------D----------	DRYPIYCSGGSCFSYGMDV	WGQG
cl2B6	---llq-a----------I-R	T----	----------	FV-S--N--F-S-----	----P---A----------ET----------	DRYPIDCSGGSCFSYGMDV	WGQ
cl2B12	--n-r----------I-R	T----	----------	FV-S--N--F-S-----	----P---A----------ET----------	DRYPIDCSGGSCFSYGMDV	WG
cl3H8	-----q-----A----------	Y----	----------T	----------	----------	DGWAYYYDRNNSFPDF	WGQG
VH3DP46	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
cl2C8	--n-r----------I---	--P-Y	----------	----------M--	----------	DRYSNDNNYYYGMDV	WG
VH3DP54	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYWMS	WVRQAPGKGLEWVA	NIKQDGSEKYYVDSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR		
cl2F3	q---lq-a----------A	NF---	----------	--N-G----------	----------	SRLPWLHFDY	WG
cl2A6, 3F5	q-n-r----------G	NF--T	----T---------	--N-G----------	----------P-----M-----	SRLPWLHLDY	WG
VH4DP65	QVQLQESGPGLVKPSQTLSLTCTVSGGSIS	SGGYYWS	WIRQHPGKGLEWIG	YIYYSGSTYYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR		
cl3F8	--------A----------S------N	-D-S---	-V----------	------T--F------	-L-M----------MT----------	MSGSRSYSQYYFDS	WG

V_L Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VKI DPK9	DIQMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP	
cl2D1	----------	----------	----------	-------	----------A----------	-------QT	FGG
cl3A9	ettl----------	----------	----------	-----L-	----------S---	-----	
cl2B5	ettl----------	----------	----------	-----L-	----------	--------PT	FG
cl2A2	--v----------	----------	----------	-------	----------	--------LT	FGQ
cl3F5	--v----------	----------	----------	-------	--H----------	--------RT	FGQ
cl2B12	--v----------	----------	----------	-------	----------	--------PIT	FGQ
cl2E1	----------	----N-AN---	----E----------	-------	----------	----N-LS	FG
VKI L12[2]	DIQMTQSPSTLSASVGDRVTITC	RASQSISSWLA	WYQQKPGKAPKLLIY	KASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYNSYS	
cl2B6	--v----------V--	------N----	----------	E------	----------	---D--WLT	FGG
VKI DPK5	DIQMTQSPSSVSASVGDRVTITC	RASQGISSWLA	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQANSFP	
cl2D12	----------	--------A--	----------	P------	---------A---------N------S---F-	L-IYV--LT	FGG
cl2D4	--v----------	----------	----------	-------	----------	------RH	FGG
cl2F3	--v----------	----------	----------	-------	----------	--------YT	FGQ
VKI DPK1	DIQMTQSPSSLSASVGDRVTITC	QASQDISNYLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	QQYDNLP	
cl2C8	----------	----------	----------	-------	----------	--------LT	FGG
VK1 DPK3	AIQMTQSPSSLSASVGDRVTITC	RASQGIRNDLG	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	LQDYNYP	
cl2G1	d-v----------	----------	----------R---	-------	----------E----------A-F-	--H-S--LT	FGG
VLI DPL8	QSVLTQPPSVSGAPGQRVTISC	TGSSSNIGAGYDVH	WYQQLPGTAPKLLIY	GNSNRPS	GVPDRFSGSKSGTSASLAITGLQAEDEADYYC	QSYDSSLSG	
cl3F8	--------P---T----------	----------	-------I-------	-------	----------G---	AAW-D----WV	FG
cl3H8	----------	---N-D---------	----------	D-N----	----------	---------WV	FGG
VLII DPL11	QSALTQPASVSGSPGQSITISC	TGTSSDVGGYNYVS	WYQQHPGKAPKLMIY	EVSNRPS	GVSNRFSGSKSGNTASLTISGLQAEDEADYYC	SSYTSSSTL	
cl2A6	----------	----------Y---	----------	D-T----	--S----------	Q-WD--VV-	FGG

Table 13. Partial V_H and V_L sequences and germline gene assignment of α-BoNT/C scFv from a human immune phage antibody library.

V_H Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH3DP42	EVQLVETGGGLIQPGGSLRLSCAASGFTVS	SNYMS	WVRQAPGKGLEWVS	VIYSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
cl3F4	----lqsa----------V---S--	T---G	----------	NL----S---I-----	-------K-R--V-----N----------T-	VDWNAIGFDF	WGQ
VH3p1	EVQLVESGGGLVQPGGSLRLSCSASGFTFS	SYAMH	WVRQAPGKGLEYVS	AISSNGGSTYYADSVKG	RFTISRDNSKNTLYVQMSSLRAEDTAVYYCVR		
cl3B7	q-n-r----------S-	T-P--	----------	G--N-----D-------	--------P----------K	GIKRYSSSSLSALDI	WGQ
VH4DP64	QLQLQESGSGLVKPSQTLSLTCAVSGGSIS	SGGYSWS	WIRQPPGKGLEWIG	YIYHSGSTYYNPSLKS	RVTISVDRSKNQFSLKLSSVTAADTAVYYCAR		
cl2A1,3E8 2D2	-v----------S----------	--D----	-------E------	--FPR---------R-	---M----------S-N----------	TKMGAAEGVFDY	WG
VH4DP71	QVQLQESGPGLVKPSETLSLTCTVSGGSIS	SYYWS	WIRQPPGKGLEWIG	YIYYSGSTNYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR		
cl2A5	-----q----------N	N----	-------E------	--FPR---Y-----R-	---M---R-------S-N----------	TKMGAAEGVFDY	WGQ

V_L Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VKI DPK4	DIQMTQSPSSLSASVGDRVTITC	RASQGISNYLA	WYQQKPGKVPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISSLQPEDVATYYC	QKYNSAP	
cl3B7	----------	----------	----------	-------	----------	-------LT	FGG
VKI DPK5	DIQMTQSPSSVSASVGDRVTITC	RASQGISSWLA	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQANSFP	
cl3E8	----------L----------	Q---D--NY-N	----------M-	D------	R----------F-	-------WT	FGG
VKI DPK9	DIQMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP	
cl3F4	----------	-------T---	-----S--V--A---	---T---	----------G----------	--G-I--LT	FGG
VKI L12	DIQMTQSPSTLSASVGDRVTITC	RASQSISSWLA	WYQQKPGKAPKLLIY	KASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYNSYS	
cl2A1	--v----------	-----V-T---	----------N--S	E--N---	----------I---	------VWY	FGG
cl2D2	--v----------	--N--------	----------	-------	---L----------A---------E------	------VLT	FG
cl2A5	--v----------	-------T---	----------N---S	-------	----------L----------	------ILT	FGG

Table 14. Partial V_H and V_L sequences and germline gene assignment of α-BoNT/E scFv from a human immune phage antibody library.

V_H Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH1DP10	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS	SYAIS	WVRQAPGQGLEWMG	GIIPIFGTANYAQKFQG	RVTITADESTSTAYMELSSLRSEDTAVYYCAR		
c12A1	----q----------R------T	R---T	---H----------	------DK--------T	---F----D--------G---P---------A	YSRGYVHFDY	WGQG
c12A10	----q--------R----------T	R-T-T	----------	------DK--------S	---F---A---------G---P---------A	----------	WGQG
VH6DP74	QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWLG	RTYYRSKWYNDYAVSVKS	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR		
c12C11	-----e----------T---------L-	-DT----	----- --------	--F-M-R--F----G---	----------T----------	QPTVHGILVYYGLDV	WG

V_L Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VK1DPK9	DIQMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP	
c12A1	--v----------	----------	----------	-------	--------G----------	------LPYT	FGG
VK1DPK8	DIQLTQSPSFLSASVGDRFTITC	RASQGISSYLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	QQLNSYP	
c12A10	--vm----------V----	W----------	----------	-------	----------	-----T-LST	FGG
VK1DPK5	DIQMTQSPSSVSASVGDRVTITC	RASQGISSWLA	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQANSFP	
c12C11	----------L----------	----------	----------	-------	----------	-------IT	FGQG

Table 27. V_H and V_L sequences and germline gene assignment of α-BoNT/A scFv from a non-immune human phage antibody library.

V_H Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH3DP46	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
cl3C5/A	-----q----------	-----	----------	----------	----------	DRPNWGFAFDI	WGQG
VH3DP47	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
cl2D8/A	----v----------	-----	----------	----------	----------	GNAVAVFWVRY	WG
cl3C8/A	-----qs----------	N----	----------	T---D--------Y-R-	----------V----E-F--Q	YSYSRGFDP	WG
cl3F6/A	q-n-r----------	-I---	----------	S----------	----------	DLLDGSGAYFDY	
VH3DP49	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
cl3H3/A	-----q----------W--------	-----	----------	----------	----------	GGALTGSFDY	WG

V_L Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VKI L12a	DIQMTQSPSTLSASVGDRVTITC	RASQSISSWLA	WYQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYNSYS	
cl3C5/A	----------A--E------	---T-------	----------	K--D-Q-	----------E--------	--LI--PLT	FGGG
VLIII DPL16	SSELTQDPAVSVALGQTVRITC	QG DELRSY YAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSGNH	
cl2D8/A	nfm----------	-- -S--T- ---	------R-------	D--Y---	----------	S---N---LNVV	FGGGTK
cl3C8/A	----------	-- -S----- ---	----------L----	---I---	----------	--------LVVV	FGGGTK
cl3H3/A	q-v----------	-- -S-K-- ---	----------	-E-S---	----------	G---G--D-WV	FGGGTKL
cl3F6/A	----------	-- -S--------	----E-----I----	-------	----------	--------VV	FGGG

Table 28. V_H and V_L sequences and germline gene assignment of α-BoNT/B scFv from a non-immune human phage antibody library.

V_H Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH3DP42	EVQLVETGGGLIQPGGSLRLSCAASGRTVS	SNYMS	WVRQAPGKGLEWVS	VIYSGGS TYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
cl2A12	q---q-s----VK----------F---	-----	----T---------	------- ---------	--------A--S----------L----K	GYGSGLPYGMDV	WGQ
VH3DP47	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
cl3D7	q---q----------	----I	----------	----------	----------R	HYDSSGYYLGY	WGQ
cl3C2,3G11	q---q----------	-----	----------	----------	----------R	----------	WGQ
cl3B6	q---q----------T----------	-----	----------	----------	----------R	----------	WGQ
cl3A12,2A9 2E1	q---qq----------	-----	----------	------DY--------	--------A--S----------R	ALGLGGAFDI	WGQ
VH3DP77	EVQLVESGGGLVKPGGSLRLSCAASGFTFS	SYSMN	WVRQAPGKGLEWVS	SISSSSSYIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR		
cl2A5	----------	-----	----------	----------	----------	GIYAIDY	WG

V_L Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VLII DPL11	QSALTQPASVSGSPGQSITISC	TGTSSDVGGYNYVS	WYQQHPGKAPKLMIY	EVSNRPS	GVSNRFSGSKSGNTASLTISGLQAEDEADYYC	SSYTSSSTL	
VLIII DPL16	SSELTQDPAVSVALGQTVRITC	QG DELRSY YAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSGNH	
cl2A12	q-a----------	-- -S-K-- ---	----------A--F-	-Q-----	---------A----------G----	----T--YQVV	FGG
cl3D7,3C2, 3B6,3G11	nfm----------	-- -S---- ---	----------L----	-------	---------TP----------	S--------LV	FGG
cl3A12,2A9	-yv----------	-- -S---- ---	----------L----	-E-----	----------	H------T-LEV	FGG
cl2E1	-yv----------	-- -S---- ---	----------L----	-E-----	----------	S---------KV	FGG
cl2A	q-v----------	R- -I--G- ---	----------L----	-E-----	----------	S---T----VL	FGG

Table 29. V_H and V_L sequences and germline gene assignment of α-BoNT/C scFv from a non-immune human phage antibody library.

V_H Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH3DP46	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
c12C12,3C4, 3F7,3H11, 3H2,2C11	----qq----L--L-S----------	-H--	----------	A--TT--YTP--N-L--	----------S---D---P----------	DIPAGAVGTIDF	WGQ
VH3DP47	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
c12D12,2F6 3D5	----q------I----------	G----	----I---------	I---P-FD---------	----------S----------S-I-----	SSRYGDSYFDY	WGQG
c12A9	q---qq----------P----------	-----	----------	------DY---------	--------A--S----------R	ALGLGGAFDI	WGQ
c13B6	----v-----V----------N	N-G-H	----------	G--S----S-----M--	----------L----------	RHYGDFS	WG

V_L Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VLII DPL11	QSALTQPASVSGSPGQSITISC	TGTSSDVGGYNYVS	WYQQHPGKAPKLMIY	EVSNRPS	GVSNRFSGSKSGNTASLTISGLQAEDEADYYC	SSYTSSSTL	
c12D12,2F6	----------	----------	----------	D------	----------	------TLV	FGG
c13D5	----------	----------	----------	-------	----------	----P----V	FGG
VLIII DPL16	SSELTQDPAVSVALGQTVRITC	QG DELRSY YAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSGNH	
c12C12,3C4 3F7,3H11, 3H2,2C11	q-v----------	-- -S-K-- ---	----------F	-E-I---	---------N----------	-------FVV	FGG
c12A9	yv----------	-- -S---- ---	----------L----	-E-----	----------	H------T-LEV	FGGG
CL3B6	----------	-- -S---- ---	----------	-------	--SE---A-R-----T----------	H--------VV	FGG

Table 30. V_H and V_L sequences and germline gene assignment of α-BoNT/E scFv from a non-immune human phage antibody library.

V_H Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH3DP46	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
cl3C8	----qq----L--A----------	-H---	----------	A--TT--YTP--N-L--	----------S-------P----------	DIPAGAVGTIDF	WG
VH3DP47	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
cl3B10	q-q-v-----S-----V----------	D---T	----T---------	G---G-AIRE-------	-------K----------I	SSPFFGPIH	WG
VH3DP49	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
cl2D7	--n-r----------	Y----	----------	-------KE--------	----------T-	SGGYPDYYYGMDV	WGQ

V_L Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VK1DPK8	DIQLTQSPSFLSASVGDRFTITC	RASQGISSYLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	QQLNSYP	
cl2D7	---m----------V----	------YN---	----------	-T-----	----------G----------	-KFS---LT	FGG
VLIII_DPL16	SSELTQDPAVSVALGQTVRITC	QG DELRSY YAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSGNH	
cl3C8	q-v----------	-- -S-K-- ---	----------F	-E-I---	---------N----------	-------FVV	FGG
cl3B10	q-v---p----------F--	-- -S- - ---	----------F--	CNF-----	----------S----------	-------T-LRV	

Table 31. V_H and V_L sequences and germline gene assignment of α-BoNT/A C-fragment scFv from a non-immune human phage antibody library.

V_H Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH3DP46	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
cl2A2,B10	-----q----------	-----	----------	----------	----------	DLDYGGNAGYFDL	WGQ
cl2B1,3G6	-----q----------	-----	----------	----------	----------	NGDPEAFDY	WG
cl2E6	-----q----------	-----	----------	----------	----------	DYTANYYYGMDV	WGQG
cl3C2	-----q----------	-----	----------	----V---------E--	----------	DLAIFAGNDY	WGQG
VH3DP47	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
cl3D1	q---vq----------T----S--	-----	----------	G-----DN-H-------	----------D----------	DLGYGSGTSSYYLDY	WGQG
cl2B6	q---q----------R	-----	----------	-------Y---------	----------R	VGVDRWYPADY	WG
cl2H6	-----v----------	--G--	----------	G-T---T----------	---------N-A----------	ALQSDSPYFDL	WG
VH3DP54	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYWMS	WVRQAPGKGLEWVA	NIKQDGSEKYYVDSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR		
cl2A8	----------T-------	----T	----------	----------L-----	----------S----------T	GRGVN	WGQG

V_L Domain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VKI_L12a	DIQMTQSPSTLSASVGDRVTITC	RASQSISSWLA	WYQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYNSYS	
cl2A8	-vv----------	----------	----------	K------	----------E---S---	--A--FPRT	FGQGTKV
cl2B6	----------	----------	----------	K------	----------	---HTISRT	FGQ
VKI_L1	DIQMTQSPSSLSASVGDRVTITC	RASQGISNYLA	WFQQKPGKAPKSLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQYNSYP	
cl2B1	----------	----S--T--N	----------L---	G------	----------	L-DYNGWT	FGG
VKI_L11	AIQMTQSPSSLSASVGDRVTITC	RASQGIRNWLA	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	LQDYNYP	
cl3G6	d----------	----S-----N	------A------Y-	G------	----------	-----GWT	FG
VLII_DPL12	QSALTQPRSVSGSPGQSVTISC	TGTSSDVGGYNYVS	WYQQHPGKAPKLMIY	DVSKRPS	GVPDRFSGSKSGNTASLTISGLQAEDEADYYC	CSYAGSYTF	
cl2E6	----------	----------	------A-P--F---	--N----	---------S---------T-A----------	N-RDS-GVV	FG
VLIII_DPL16	SSELTQDPAVSVALGQTVRITC	QG DELRSY YAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSGNH	
cl2B10	q-v---------S----------	-- -S---- ---	----------L----	-E-----	----------	---------QV	FGG
cl2A2	----------	-- -S---- ---	--------T-L--M-	-E----Q	----------E----------	H-----VT-LR	FGGGTK
cl3D1	----------	-- -S---- ---	------E---L----	-V-----	---------N----------	---------VV	FGGGTKL
cl2H6	----------L--------S---	-- -S--T- ---	----------L----	-------	---------YP----------	CN--------VV	FGG
cl3C2	----------	-- -S--------	-I----R---I--MF	--D----	----------	K----R---LAL	FGGGT

Table 38. Deduced protein sequences of heavy and light chain variable region of BoNT/A H_C binding scFv classified by epitope recognized

Heavy Chains

Clone	Lib	Framework 1	CDR 1	Framework 2	CDR 2	Framework 3	CDR 3	Framework 4
Epitope 1								
C15	1	QVKLQQSGAELVRPGASVKLSCKTSGYSFT	SYWMN	WVKQGPGQGLEWIG	MIHPSNSEIRFNQKFED	MATLTVDKSSSTAYMQLSSPTSEDSAVYYCAR	GIYYDYDGGNYYAMDY	WGQGTTVTASS
C9	1	----------	-----	----------	----------N	----------	----V----------	--------V--
1D5	2	E---VE----------N----A------	-----	----R---------	-------T-L----K-	K----------	-------E-Y--TL--	------L-V--
C1	1	----------A------	-----	----R---------	-------DT--------	K-----R-----IH----------	-L-GYGF WYFDV	--------V--
S25	1	----------A----L-	-----	----R---------	-----D-DT--------	K------T----------	-L-NGF WYF-V	--------V--
1B6	2	--Q----------V---I---A---T-I	D-A-H	-----S-AKS-----	V-SSYYGDTDY---I-KG	K---------N----E-ARL--D---I-----	RGKG -----	--------V--
1C9	2	--Q-K---------V---I---G---T-I	D-AVH	----SHAKS-----	V-STYYGDADY-PK-KG	K-----N---N----E-PRL------I-----	RGKG ----	-----S--V--
1E8	2	E-Q--E--PG--K-SQ-LS-T-TVT---I-	D-AW-	-IR-F--KK---M-	Y-S YSGSTGYNPSIKS	RISI-R-T-KNQFFL--N-V-T--TGT-----	-YD ----	-----S--V--
1G7	2	E-Q--E--PG -K-SQ-LS-T-TVT---I-	D-AWY	-IR-F--KK---M-	Y-S YSGSTGYNPSIKS	RISI-R-T-KNQFFL--N-V-T--TGT-----	-YD ----	-----S--V--
Epitope 2								
1A1	2	EVKLVESGGGLVQPGGSRKLSCATSGFTFS	DYYMS	WIRQSPDKRLEWVA	TISDGGTYTYYPDSVKG	RFTISRDNAKNTLYLQMSSLKSEDTAMYYCVR	HGYGNYPSH WYFDV	WGAGTTVTVSS
1F1	2	----------L-----A------	N-G--	-V---T---------	M--S--S-N--S-----	-V---------S---------Q-------L-T-	--------Y -----	----------
C39	1	Q-Q-Q-----S-K----L-----A------	-----	-V---T-E-------	------S---------	----------N----------I-----	YR-DEGL -Y	--Q--------
C25	1	Q-Q-Q-------K----L-----A------	----Y	-V---T-E-------	------S---------	----------N----------S-	YR-DDAM -Y	--Q--------
2G5	2	----------K----L-----A------	S-A--	-V---T-E-------	----------T-N---	----------HN------H----------A-	NLPYDHV -Y	--Q---S-----
3C3	2	-----K-------K----L-----A------	S-A--	-V---T-E-------	----------T-N---	----------HN------H----------A-	NLPYDHV -Y	--Q---S-----
3F4	2	EG----------K----L-----A------	S-A--	-V---T-EH------	-------F---T-N---	----------HN------H----------A-	NLPYDHV -Y	--Q---S-----
3H4	2	----------K---PL-----A------	S-A--	-V---T-EH------	-------F---T-N---	----------HN------H----------A-	NLPYDHV -Y	--Q---S-----
Epitope 3								
1B3	2	EVQLQESGGGVV*PGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	DWSEGYYYYG MDV	WGQGTTVTVSS
1C6	2	QI---IQ----------	-----	----------	----------	----------	--------- ---	----------
2B6	2	VKLVESGP-L-KPSQSLSLTCTVTGYSIT-	D-AWN	-I---F---NK---MG	Y-N-----N-NP -L-N	-ISIT--T---QFF-KL--VTS----T-----	AGDGY-VD WYFDV	---T--------
1G5	2	Q----Q---AEL----A-VKM--K---Y--T	---WIT	---K-R--Q----IG	D-YPGSGSTNYNEKF-S	KA-LTV-T-SS-A-M-LS--TS--S-------	ELGD A--Y	-----S-----
1H6	2	-----Q---AEL-K---A-VKM--K---Y--T	---WIT	---K-R--Q----IG	D-YP-SGSTNYNEKF-S	KA-LTV-T-SS-A-M-LS--TS--S-------	ELGD A--Y	-----S-----
Epitope 4								
1F3	2	EVQLQQSGAELVKPGASVKLSCKASGYTFT	SFWMH	WVKQRPGRGLEWIG	RLDPNSGETKYNEKFKS	KATLTVDKPSSTAYMELSSLTSEDSAVYYCAR	EAYGYWN FDV	WGTGTTVTVSS
2E8	2	----------	-----	----------	----------K----	----------	------- ---	----------

Light Chains

Clone	Lib	Framework 1	CDR 1	Framework 2	CDR 2	Framework 3	CDR 3	Framework 4
Epitope 1								
C15	1	DIELTQSPAIMSASPGEKVTMTC	SASS SVSHMY	WYQQKPGSSPRLLIY	DTSNLAS	GVPIRFSGSGSGTSYSLTISRMEAEDSATYYC	QQWSSYPFT	FGSGTKLEIKR
C9	1	--D---------S-------I--	---- ---Y-H	-F-----T--KPW--	S------	---A----------SV----A-----	--Y-G--L-	--A-----I--
1D5	2	----------A--------I--	----S I-S-NLH	-----SETSPKPW--	G------	---V----------S-----A-----	---G---L-	--G-----I--
C1	1	----------	---- ---Y--	----------	-------	---V----------A-----	-------L-	--A--------
S25	1	---------L-A--------I--	-V--S I-S-NLH	-----S-T--KPW--	G------	---V----------S-----A-----	-------L-	--A-----I--
1B6	2	---------SIAV-L-QRA-IS-	RA-ESVDSYGN-F-H	-------QP-K-----	RA---E-	-I-A-------R-DFT---INPV--D-V-----	*-SNED-P-	--A--------
1C9	2	---------SIAV-L-QRA-IS-	RA-ESVDSYGN-F-H	-------QP-K-----	RA---E-	-I-A-------R-DFT---NPV--D-V-----	--SNED-Y-	--G-----I--
1E8	2	----------	---- ---Y-H	-----S-T--KRW--	---K---	---A----------S-----A-----	-----N-L-	--A--------
1G7	2	----------	---- ---Y-H	-----S-T--KRW--	---K---	---A----------S-----A-----	-----N-L-	--A--------
Epitope 2								
1A1	2	DIELT*SPASLAVSLGQRATISC	RASESVDSYGNSFMH	WYQQKPGQPPKLLIY	LASNLES	GVPARFSGSGSRTDFTLTIDPVEADDAATYYC	QQNNEDPYT	FGGGTKLEIKR
1F1	2	-----Q--T----------	----------	----------	-------	---------A-----	---------	----------
C39	1	-----Q---------R------	----------H-----	----------	-------	---------A-----	---------	----------
C25	1	-----Q----------	---------H---Q	----------	R-----P	-I---------G-------N------V-----	--S----F-	--S---------
2G5	2	-----Q---IMSA-P-EKVTIT-	S--S SV-Y---	-F-----TS-K-W--	ST---A-	----------G-SYS---SRM--E-A-----	--RSSY---	----DQAGN-S
3C3	2	-----Q---IMSA-P-EKVTIT-	---------H---Q	-F-----TS-K-W--	ST---A-	----------G-SYS---SRM--E-A-----	--RSSY---	----DQAGN--
3F4	2	-T---Q---IMSA-P-EKVTMT-	S--S SV-Y-Y	-------SS-R-----	DT---A-	---V--------G-SYS---SRM--E-A-----	--WSSY-P-	----------
3H4	2	-----Q---IMSA-P-EKVTMT-	---S- VSS-YL-	-------SS-R-----	DT---A-	---V--------G-SYS---SRM--E-A-----	--WSSY-P-	----------
Epitope 3								
1B3	2	DSELTQSPTTMAASPGEKITITC	SASSS ISSNYLH	WYQQRPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPRT	FGGGTKLEIKR
1C6	2	-I-------ASL-V-L-RRA--S-	R--E-VEYYGTSLMQ	----K---QP------	AA--VE-	----------DF--N-HPV-E -I-M-F-	--SRKV-W-	----------
2B6	2	YI-------ASL-V-L-QRA--S-	R--E-VDSYGNSFM-	----K---QP------	LA---E-	----------R-DFT---DPV--D-A-----	--NNED-Y-	----------S
1G5	2	-I-------ASL-V-L-QRA--S-	R--E-VEYYGTSLMQ	----K---QP------	AA--VE-	-A----------DF--N-HPV-ED-I-M-F-	--SRKV-Y-	----------
1H6	2	-I-------AI-S-------V----	-V--- ---SN--	----KS-T----W--	G------	---V----------SS-----A-----	--W--Y-L-	--A---V-LR-
Epitope 4								
1F3	2	DIELTQSPASMSASPGEKVTMTC	RATSS VSSSYLH	WYQQKSGASPKLWIY	SASNLAS	GVPSRFSGSGSGTSYSLTISSVEAEDAATYYC	QQYIGYPYT	FGGGTKLEIKR
2E8	2	--------TT-A------I-I--	S-S-- IG-N---	-----P-F----L--	RT-----	---A----------GAM----V-----	--GSSI---	----------

Full length sequence not determined for clones C12, C13, C2, and S44 (all bind epitope 1). Accession can be made through GenBank with numbers AF003702 to AF003725.

Appendix Four

Bibliography of Publications and Abstracts

Publications:

1. Chen F, Kuziemko G, Amersdorfer P, Wong C, Marks JD and Stevens RC. Antibody mapping to domains of Botulinum serotype A in the complex and uncomplexed form. Infection Immunity. 65: 1626-1630, 1997.

2. Amersdorfer P, Wong C, Chen S, Smith T, Deshpande S, Sheridan R and Marks JD. Molecular characterization of the murine immune response to Botulinum neurotoxin type A binding domain as assessed using phage antibody libraries. Infection Immunity. 65: 1997.

3. Sheets MD, Amersdorfer P, Finnern R, Sargent P, Lindqvist E, Schier R, Hemingsen G, Wong C, Gerhart JC and Marks JD. Efficient construction of a large non-immune phage antibody library: the production of panels of high affinity human single chain antibodies to protein antigens. Proc. Natl. Acad. Sci. USA. 95: 6157-6162, 1998.

Submitted manscripts

1. Powers D, Amersdorfer P, Poul M-A, Shalaby MR, Adams GP and Marks JD. Expression and characterization of single-chainFv-Fc fusions in Pichia pastoris. Submitted.

Appendix Five

List of Personnel Receiving Pay From This Effort

James Marks M.D., Ph.D.
Peter Amersdorfer Ph.D.
Steven Chen
Ricarda Finnern Ph.D.
Hanna Merritt
Robert Schier Ph.D.
Cindy Wong
Mark Wahrenbrock
Shirley Wu